Cultivation of a free-living nematode *Panagrolaimus* sp. in batch and fed-batch liquid culture of *Saccharomyces cerevisiae* for larval food in marine aquaculture

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Summary. The free-living nematode *Panagrolaimus* sp. (strain NFS-24-5) has potential to be used as live food for early stages of marine aquaculture fish and shrimps. One prerequisite for commercial application is the mass production at low costs. The objective of this study was to test whether fed-batch conditions can increase nematode yields of monoxenic liquid cultures with cells of the yeast *Saccharomyces cerevisiae* in flasks and bioreactors. Liquid cultures in flasks observed over 16 days provided contradicting results. In lab-scale bioreactors significantly lower nematode yields were obtained under fed-batch conditions. Maximum nematode density reached in a bioreactor process under batch conditions was 270×10^3 nematodes ml^{-1} and in fed-batch at 153×10^3 nematodes ml^{-1}. In flasks nematodes had a lag phase of 8 days until reproduction started. This period was reduced to 4 days by culturing in bioreactors.

Key words: Bioreactor, flasks, monoxenic culture, live food, strain NFS-24-5.

Production of living food organisms for early life stages of marine fish and crustaceans species in aquaculture accounts for a significant proportion of the overall production costs (Fisher & Fletcher, 1995; Wilkenfeld *et al.*, 1984). Larviculture is considered to limit the growth of the aquaculture sector (Dhert *et al.*, 2001). The most common organisms used as live food are *Artemia nauplii* and rotifers (Dhert *et al.*, 2001; Figueiredo *et al.*, 2009). *Artemia* cysts are harvested from hypersaline lakes and are hatched when needed within 24 h. Hatching rates and nutritional quality can vary, as well as the amounts harvested (Lavens & Sorgeloos, 2000; Kolkovski, 2001). Production of rotifers requires facilities and skilled personal and they cannot be stored. Due to these problems research has targeted on finding alternative food organisms (Santiago *et al.*, 2004).

Free-living nematodes can be an alternative and do not have most of those disadvantages. Promising results have been reported for the use of the nematode *Panagrellus redivivus* (Rhabditida: Panagrolaimidae). Several fish species have been fed with this nematode, like carps, *Cyprinus carpio* (Schlechtriem *et al.*, 2004a, b), *Hypophthalmichtys nobilis* (Rottmann *et al.*, 1991), *Aristichthys nobilis* (Santiago *et al.*, 2003, 2004), ornamental catfish *Synodontis petricola* (Sautter *et al.*, 2007) as well as the penaeid shrimp species *Penaeus aztecus*, *P. setiferus* (Wilkenfeld *et al.*, 1984), *Litopenaeus vannamei* (Biedenbach *et al.*, 1989; Focken *et al.*, 2006) and *P. indicus* (Fisher & Fletcher, 1995; Kumlu *et al.*, 1998; Kumlu, 1999). However, *P. redivivus* cannot be desiccated and storage and transportation of such nematode material would be difficult and costly.

In a screening for potential candidates among free-living nematode species, *Panagrolaimus* sp., strain NFS-24-5 (Rhabditida: Panagrolaimidae) was chosen as a food organism for early stages of marine fish and shrimp larvae. It displayed several favorable characteristics: a suitable size and high reproduction rate, it remained active in sea water for over 24 h and survived moderate desiccation (Honnens *et al.*, 2013). Desiccation tolerance facilitates product formulation, storage and transport. However, the lack of competitive methods for mass production prevented the use of nematodes

as live food on a commercial scale (Schlechtriem *et al.*, 2004b).

Mass production of bacteria, yeasts and fungi is usually performed in liquid culture in large scale bioreactors. As rhabditid nematodes feed on micro-organisms, such technology has also been developed to produce insect pathogenic nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos on their symbiotic bacteria *Photorhabdus* Boemare, Akhurst & Maurant and *Xenorhabdus* Thomas & Poinar, respectively, for use in biological control of pest insects. Most commercially available biocontrol nematode products are produced in liquid culture, which can reach volumes of up to 60 m³ in scale (Ehlers, 2001). Knowledge obtained for cultivation of biocontrol nematodes might also be applicable to the culture of *Panagrolaimus* sp. strain NFS-24-5.

The feasibility to propagate *Panagrolaimus* sp. in monoxenic liquid culture on baker's yeast *Saccharomyces cerevisiae* was recently evaluated (Honnens & Ehlers, 2013). Yields in terms of nematode numbers as well as biomass were highly variable. The maximum number of nematodes varied from 45000-238000 ml^{-1} and maximum biomass from 49 to143 g l^{-1}. The size spectrum of *Panagrolaimus* sp. individuals ranged from 176 × 8 µm to 1377 × 61 µm and the wet weight from 8.15 to 3202.39 ng. Water content of the nematodes was 71.7 ± 2.5%, so dry weight per individual was 2.31 to 905.95 ng. Based on the data on size and dry weight per individual, it was concluded that *Panagrolaimus* sp. strain NFS-24-5 has a potential as a substitute for rotifers as live food organism.

In the previous experiments with *Panagrolaimus* sp. (Honnens & Ehlers, 2013), the density of yeast cells decreased during the experiment to low levels. As food density has a pronounced effect on fecundity of nematodes (Schiemer *et al.*, 1980; Schiemer, 1982), the limited availability of yeast cells towards the end of the cultivation process has probably limited nematode reproduction. Therefore, the objective of this investigation was to test fed-batch conditions *vs* batch production to increase nematode yields to develop an economically more competitive production process. The experiments were conducted in flasks as well as lab-scale bioreactors.

MATERIALS AND METHODS

Culture of *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* axenic cultures were established from commercially available baker's yeast (DHW Vital Gold, Deutsche Hefewerke GmbH, Nürnberg, Germany) by suspension and dilution in sterile water and streaking on a YM-Agar plates (DSMZ, 2007) (all ingredients purchased from Carl Roth GmbH, Karlsruhe, Germany: 3 g yeast extract, 3 g malt extract, 5 g soy peptone, 10 g glucose, 10 g agar, 1 l distilled water). Single colonies were picked and *S. cerevisiae* was then propagated in 100 ml liquid YM medium in 500 ml Erlenmeyer flasks. The flasks were then incubated in the dark at 25°C on an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 190 rpm for 24 h. Stocks were stored at –80°C after addition of 15% glycerol (Roth).

Suspensions of *S. cerevisiae* for fed-batch feeding experiments were prepared from baker's yeast of the same brand. One package of 42 g fresh weight was suspended in 50 ml sterile Ringer's solution (all ingredients purchased from Roth: 9 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, 0.2 g $NaHCO_3$, distilled water to 1 l) to obtain single cells.

Production of monoxenic *Panagrolaimus* sp. cultures. Strain NFS-24-5 of *Panagrolaimus* sp. was used (Honnens & Ehlers, 2013). Axenic cultures were produced by incubating nematodes for 48 h in a suspension of antibiotic and antimycotic compounds containing 1 mg l^{-1} natamycin (Natajen-S; Jeneil Bioproducts GmbH, Schechen, Germany), 100 mg l^{-1} sodium-ampicillin (Roth) and 100 mg l^{-1} streptomycin sulphate (Serva GmbH, Heidelberg, Germany). Monoculture was checked by observation under a microscope at × 1000 magnification (Axioskop, Zeiss, Germany) and by plating dilutions on tryptone-soy-agar (Roth) and YM-agar plates after incubation for 24 h. Axenic nematode and axenic *S. cerevisiae* cultures were combined to establish monoxenic nematode pre-cultures.

Batch and fed-batch liquid cultures in flasks. *Panagrolaimus* sp. was propagated in the liquid medium OM4 (all ingredients purchased from Roth, except were indicated: 2 g glucose, 4 g yeast extract, 7 g defatted soy flour (Cargill, Gent, Belgium), 3 g lecithin (Cargill), 30 g rapeseed oil (Raiffeisen, Kiel, Germany), 5 g NaCl, 1 g KCl, 0.5 g $CaCl_2$, 1 g $MgSO_4 \times 7H_2O$, 3.4 g KH_2PO_4, 1.7 g K_2HPO_4, 1 l distilled water). Ten 500 ml-Erlenmeyer flasks were filled with 100 ml of OM4, closed with cotton plugs and then autoclaved. After cooling, 3 ml of a *S. cerevisiae* culture was added to each flask and the flasks were then incubated at 25°C in the dark on a rotary shaker at 190 rpm for 24 h. Then nematodes from monoxenic cultures (mixed stage populations dominated by first-stage juveniles) were inoculated to obtain an average density of 5000 nematodes ml^{-1}. The flasks were then incubated at 190 rpm and 25°C

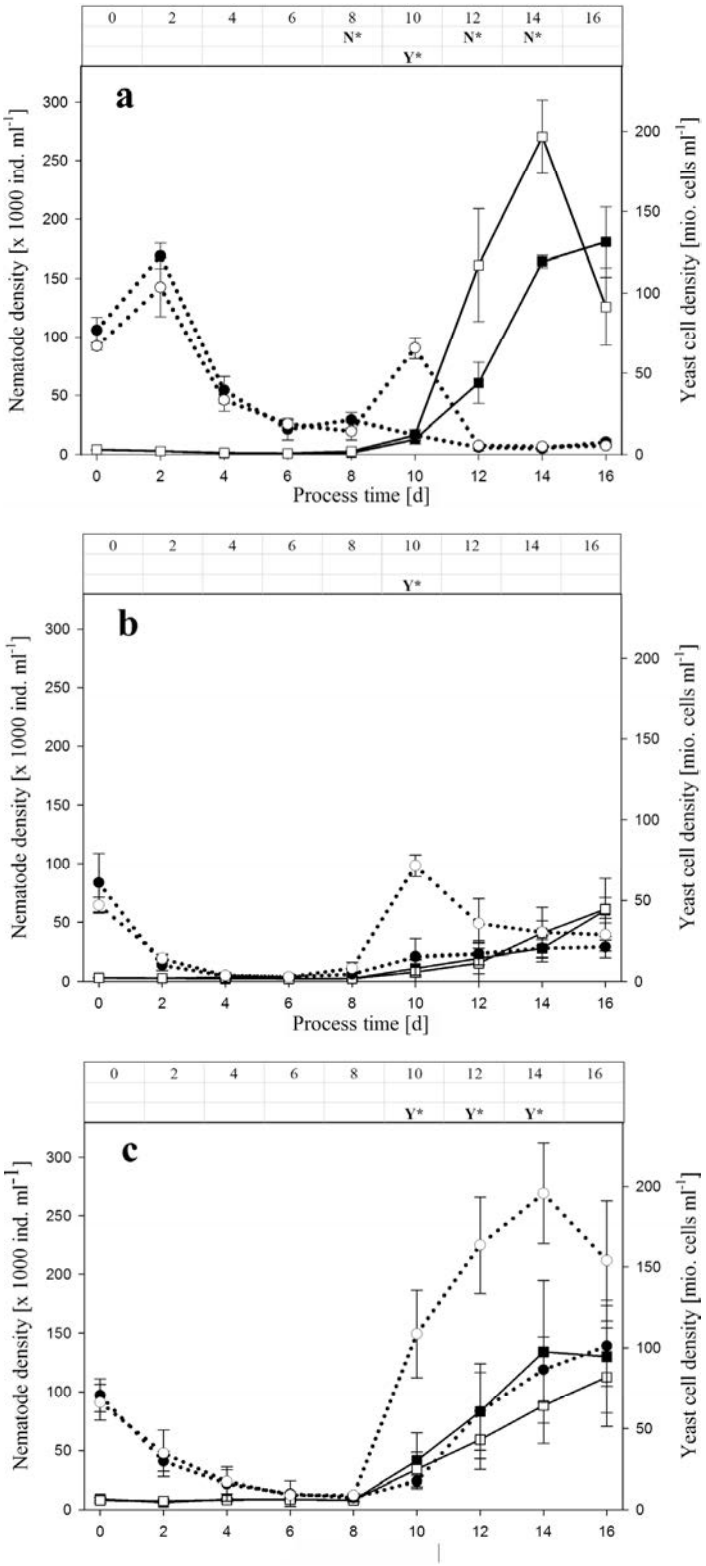

Fig. 1. Mean ± standard deviation of nematode (solid line) and yeast cell density (dashed line) over cultivation of 16 days comparing batch (white symbols) and fed-batch (black symbols) conditions of three experiments (a, b and c). Fed-batch flasks received additional yeast cells at day 8. Significant differences in yeast cell density (Y) or nematode numbers (N) at the same day between batch and fed-batch are indicated above the figure with asterisks. Experiments a and c consisted of 5, experiment b of 4 flasks.

for 16 days. Nematode and yeast cell density were recorded every second day. Half of the flasks received 5 ml of *S. cerevisiae* suspension on day 8 after inoculation of yeast cells (fed-batch). All handling was conducted under aseptic conditions. The experiment was conducted three times. In the second experiment only 4 flasks per group were evaluated due to contamination of 2 flasks.

Assessment of nematode and yeast cell density. Aliquots of the nematode suspension were thoroughly mixed to ensure even distribution and then diluted with Ringer's solution. A defined amount of the diluted suspension was transferred to square cell well chambers subdivided in 16 squares by an elevated grid (Bio-one Cellstar®; 384 well cloning plate; Greiner, Solingen). Counting was conducted under a dissection microscope (Axiovert 25, Zeiss, Germany) at × 50 magnification. Two samples were drawn and diluted; each of them was counted twice and the arithmetic mean was calculated.

The yeast cell density was assessed by counting cells in a Thoma-type chamber (0.01 mm depth) under a microscope at × 400 magnification (Axioskop, Zeiss, Germany). Samples were withdrawn from the flasks and fixed by addition of the identical amount of 10% acid-free phosphate-buffered formaldehyde (Roth) and thorough mixing. To correct for the uneven distribution of cells in the medium that contains particulate matters, two samples were taken, each sample was counted twice and the average value was used.

Batch and fed-batch cultivation in lab scale bioreactors. Bioreactors of 20 l medium volume (model Techfors®S, Infors GmbH, Einsbach, Germany) were used, equipped with Pt-100 temperature sensor, pressure sensor, mass flow controller (all from Infors), saturated oxygen-probe (InPro® 6800, Mettler-Toledo GmbH, Gießen, Germany) and pH-sensor (Mettler-Toledo). Online documentation and control was done with the IRIS NT® software (Infors AG, Bottmingen, Switzerland). The bioreactors were equipped with Rushton-type turbines and central cylinder to produce internal flow conditions in the vessel. A ring sparger was situated below the turbines at the bottom of the vessel and four baffles were situated at the sides.

The OM4 medium was used, supplemented with 0.5% antifoaming agent (silicone emulsion Silfoam SE2 Wacker Chemie AG, München). Yeast cultures were inoculated at 3% medium volume and cultivated for 24 h. Then nematodes were inoculated to obtain start densities of approximately 3.000 nematodes ml^{-1}. During the cultivation the temperature was set at 26°C controlled by addition of cool or heated water into the double jacket of the bioreactors. The pressure was kept at 0.1 bar above atmospheric pressure. The dissolved oxygen concentration (pO_2) was regulated by cascade-mode with adjustment of aeration rate (0-40 l min^{-1}) at first and agitation speed at second stage (200-600 rpm) to reach a set point value of 20% (range 15-30%) of medium saturation concentration. Assessment of nematode and yeast cell density was carried out as described above. Of 15 bioreactor runs conducted, 10 were run under fed-batch conditions adding *S. cerevisiae* suspensions. Feeding was done earliest 5 days after process start (with one exception on day 2) with 16.8-33.6 g yeast l^{-1} fermentation broth. Two fed-batch cultures received an addition 33.6 g yeast l^{-1} two days after the first feeding.

Statistics. Data on yeast cell and nematode density from flask-cultures were assessed for normal distribution with the Shapiro-Wilk test ($P = 0.05$). Since data were not normally distributed in some groups, a non-parametric test was chosen to check for significant differences. Batch and fed-batch groups of the same organism (yeast or nematode) on the same day were compared by the Mann-Whitney Rank Sum Test ($P = 0.05$). Calculations were conducted with SigmaPlot 11.0 (©Systat Software Inc., San José, USA).

Data from bioreactor-processes on nematode density at 230 h after inoculation was determined by linear interpolation between the measured values. The density at 230 h of batch and fed-batch was then compared by unpaired t-test after check for normal distribution by the Shapiro-Wilkinson test ($P = 0.05$) and equal variances test.

RESULTS

Batch and fed-batch in flasks. Although culture flasks of all three experiments were started with comparable yeast and nematode densities, the results differed considerably. The nematode density hardly changed from inoculation until day eight in all three experiments and then started to increase. In experiment 1 a pronounced increase to maximum average density of 270500 nematodes ml^{-1} in the fed-batch flasks on day 14 and 180667 on day 16 in the batch flasks was observed (Fig. 1A). On day 16, the mean density in the fed-batch flasks had dropped below that of the batch cultures. Significant differences were recorded between batch and fed-batch on days 8 ($P = 0.016$), 12 and 14 ($P = 0.008$). In experiment 2 no significant differences between fed-batch and batch culture was recorded with mean maximum densities on day 16 of 60300 ml^{-1} in fed-

batch and 61267 ml^{-1} in batch cultures (Fig. 1B). In experiment 3 the nematode density in batch was higher than in fed-batch but the differences were not significant. The highest average values were obtained on day 14 in the batch flasks (134133 nematodes ml^{-1}) and on day 16 in the fed-batch flasks (112587 nematodes ml^{-1}) (Fig. 1C).

Although yeast cell densities at the beginning of the three experiments in flasks were all at approximately 65 × 10^6 cells ml^{-1} (Fig. 1), the densities developed differently in the three experiments. Only in the first experiment (Fig. 1A) did the density increase until day two and then decrease in a similar manner to that recorded in experiments 2 and 3 (Figs 1B and 1C). In both fed-batch and batch cultures in all three experiments the yeast cell density declined until day 8. Feeding in fed-batch increased the density on day 10 in all three experiments significantly (P = 0.008 in experiments 1 and 3; P = 0.029 in experiment 2). But whereas it declined again in experiments 1 and 2, the density increased in experiment 3 in fed-batch as well as in batch cultures. In fed-batch cultures the increase was more pronounced with significant differences to the batch cultures on day 12 and 14 (both P = 0.008). The yeast cell densities reached values below 10 × 10^6 cells ml^{-1} in experiment 1, to a final level of 20-30 × 10^6 cells ml^{-1} in experiment 2 and to 100 × 10^6 cells ml^{-1} in experiment 3 (Fig. 1).

Batch and fed-batch bioreactors. The yeast cell density was higher in fed-batch than in batch cultures (Fig. 2). Nematode density after 10 days of

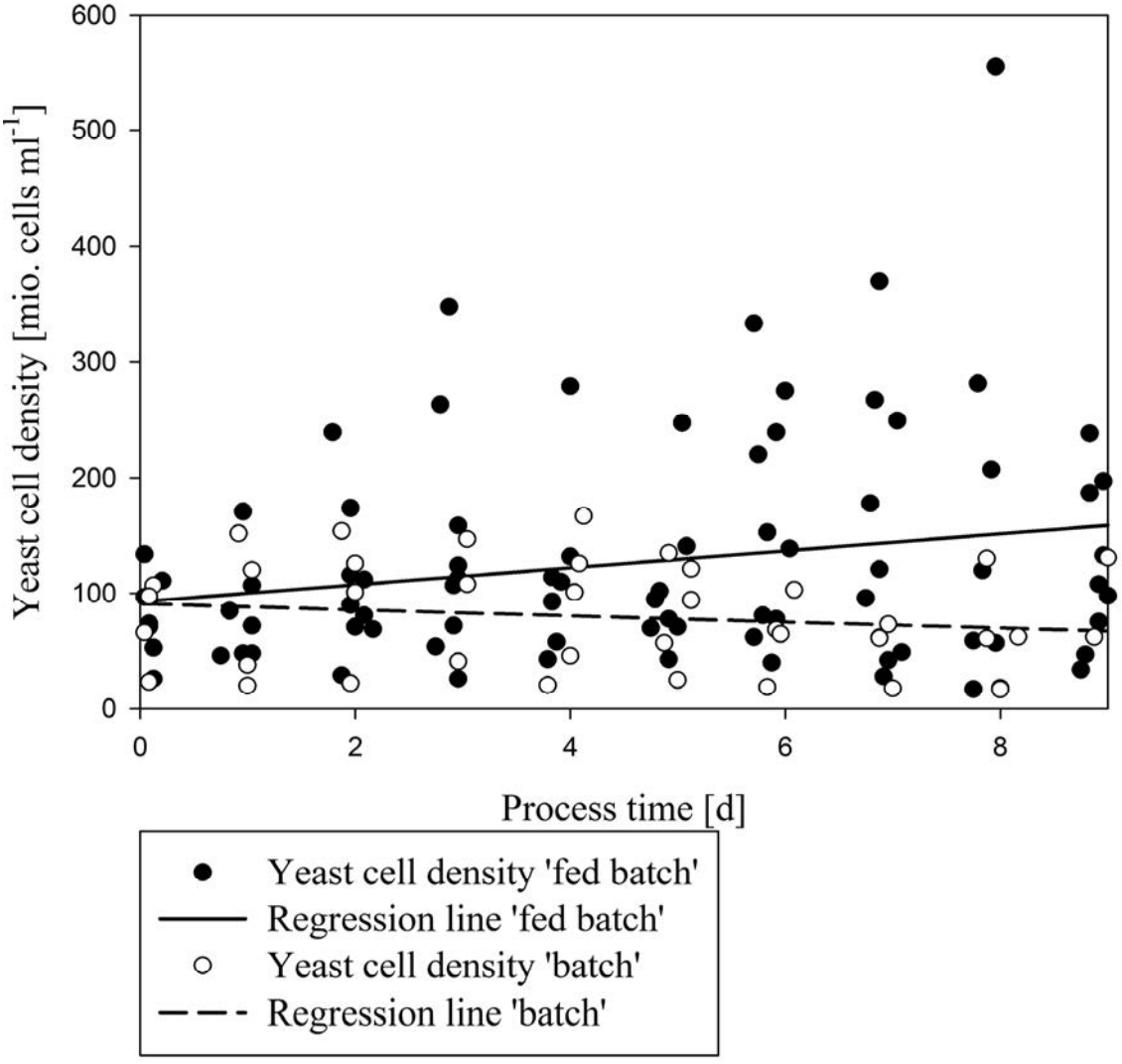

Fig. 2. Yeast cell density recorded in batch and fed-batch bioreactor cultures and regression lines.

cultivation in bioreactors was significantly lower in fed-batch than in batch cultures ($P = 0.047$) (Table 1). Nematode populations began to increase 4-7 days after inoculation (Fig. 3). Maximum density was reached in a bioreactor process under batch conditions with 270×10^3 nematodes ml^{-1} (Fig. 3A) and maximum in fed-batch at 153×10^3 nematodes ml^{-1} (Fig. 3B).

Table 1. Nematode density (mean ± standard deviation) 10 days after cultivation in bioreactors under fed-batch and batch conditions.

Feeding yeast	N	Nematode density ($\times 10^3$ ml^{-1})	
		Mean	Standard deviation
Fed-batch	10	63	39
Batch	5	128	79

DISCUSSION

Fed-batch is an appropriate method to increase cell density of micro-organisms during liquid culture processes. The results of this investigation, however, indicate that an addition of yeast cells during the monoxenic liquid culture of the nematode *Panagrolaimus* sp. (strain NFS-24-5) was not able to increase the yields of this nematode.

Schiemer (1982) working on *Caenorhabditis briggsae* divided food densities into three ranges depending on their effect on nematode growth: first, a food concentration below a maintenance level not allowing reproduction; second, a food concentration exceeding maintenance level with strongly increasing offspring production of the nematodes with increasing with food supply; and, third, a surplus food supply, resulting in only a weak increase with additional food supply. In the present experiments, the yeast cell density was not growth limiting for the nematodes either in flask or in bioreactor batch cultures and additional food supply also did not increase reproduction. Somatic growth and multiplication were observed in flask and bioreactor cultures in batch and fed-batch cultures, but no relation of the nematode density with the yeast density was recorded. On the contrary, increasing the yeast cell density by fed-batch conditions resulted in fewer nematodes than in batch cultures in bioreactors. Whereas experiments in flask provided contradicting result, the bioreactor experiments clearly indicated the negative effect of fed-batch conditions on the nematode propagation.

One possible assumption is that process factors, like oxygen saturation, have been influenced by the addition of the yeast cells and have limited nematode multiplication. Nematodes other than yeast cells need a continuous supply of oxygen. The documentation of the bioreactor process parameters indicated that addition of yeast cells always resulted in a sharp decline of saturated oxygen concentration in the growth medium; however, the effect never lasted longer than a few minutes. When saturated oxygen concentration was below the set point, the stirrer speed and air flow automatically increased and oxygen saturation rapidly reached set values again. Should oxygen saturation have been a problem, then flask cultures would have suffered from this effect much more than bioreactor cultures, as oxygen saturation is not controlled and shaker speed was constant in batch and fed-batch flasks. However, one flask experiment had higher nematode yields in fed-batch then in batch cultures, so reduced oxygen levels were most probably not the cause for lower yields.

It cannot be ruled out that addition of yeast cells influenced nematode biomass instead of number. Dos Santos (2008) observed in two *Diplolaimelloides* species that additional energy taken up at higher food densities resulted in larger somatic growth but production of fewer offspring. However, in the presented investigation the biomass of the nematode population was not assessed and consequently no conclusion can be made about whether high yeast cell density caused a larger biomass of the nematode population.

Differences in nematode yield between batches were pronounced and variation between experiments was high. It is likely that the variability between batches is at least in part caused by differences in the stage composition in nematode inocula. Although it is usually dominated by first-stage juveniles the proportion and age of other stages differed between inoculum batches. Nematodes of different sizes have different feeding rates (Marchant & Nicholas, 1974).

In flask cultures a prolonged lag phase of 8 days was observed. This was also reported in previous experiments with flask cultures of the same strain (Honnens & Ehlers, 2013). Nematode body size was measured and data indicated that for the first four days the nematodes did not grow as they did in the present experiments in flasks. It was suspected that the nematode might have been unable to feed on fully grown yeast cells when in the first juvenile stage. Yeast cells multiply by budding and after cell division resulting cells are smaller. So growth might have started only after the availability of smaller cells. This reason can now be excluded as this prolonged lag phase was not recorded in bioreactor

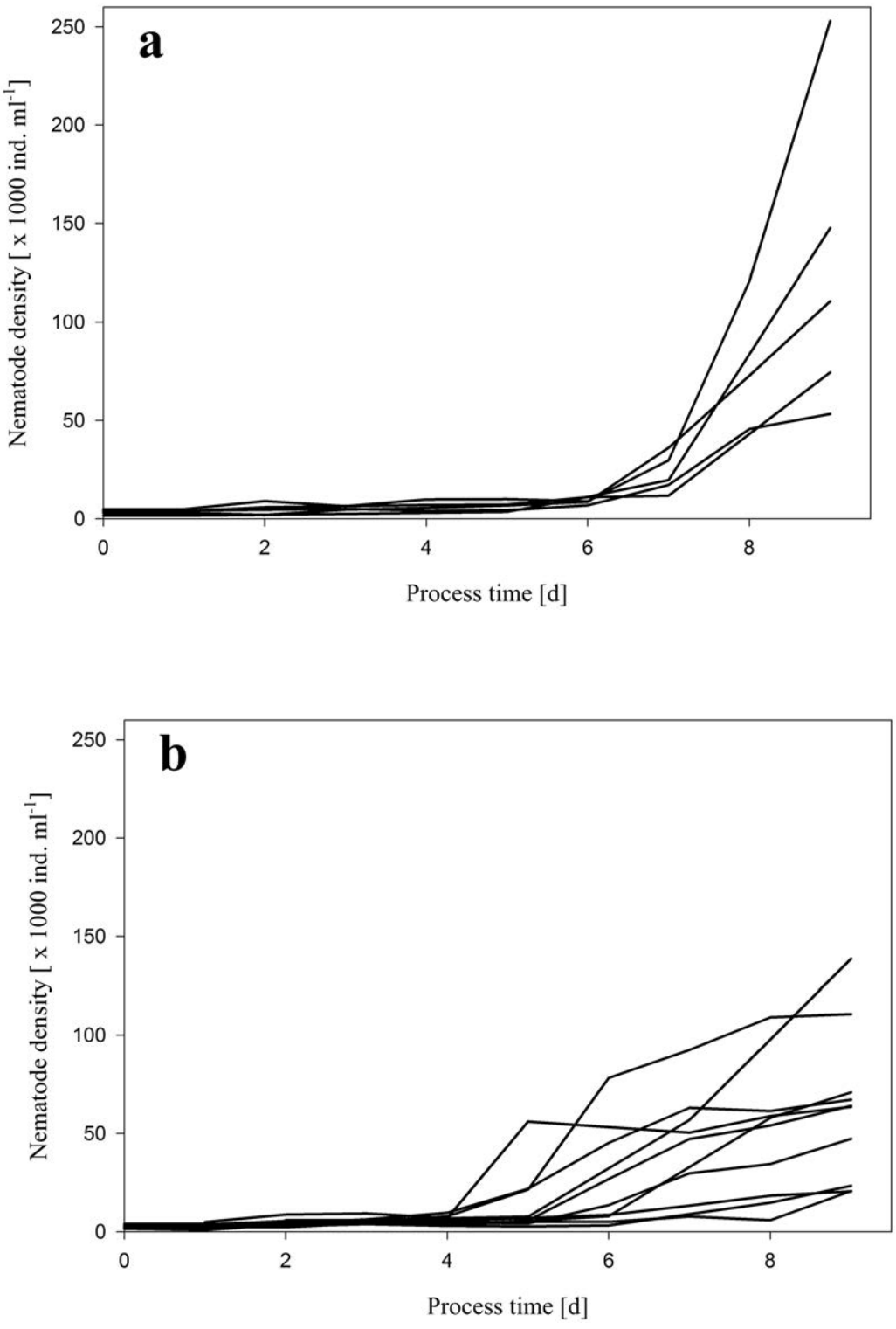

Fig. 3. Nematode population density in bioreactors recorded in batch (a; n = 5) and fed-batch (b; n = 10) cultures.

cultures in which growth and reproduction started after a lag phase of 4 days. The reason for the prolonged lag phase in flask cultures might have been caused by too high yeast cell density, which produced lack of oxygen in flasks but not in bioreactor cultures.

The prolonged lag phase observed in flasks would have been a reason for exclusion of this nematode strain from commercial production for food of marine shrimps and fish larvae as process time is a major cost factor in commercial production (Ehlers, 2001). Obviously, the process time can be substantially reduced by cultivation in bioreactors. The cultivation time of 14-15 days until maximum nematode numbers observed in flask cultures was reduced by several days and process time is closer to a time span recorded also for the commercial production of entomopathogenic nematodes, which is usually within 10 days (Ehlers, 2001).

The results of this investigation indicate that the process technology is still far from commercial exploitation, which would need predictable yields and more homogenous population development (Ehlers, 2001). However, the results have demonstrated that liquid culture of *Panagrolaimus* sp. can be successfully scaled up to lab-scale bioreactors and can reach considerable densities. If future research is able to further stabilise process technology for this nematode, production at competitive costs might be possible to supply marine aquaculture with live food organisms based on *Panagrolaimus* sp.

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Резюме. Показан значительный потенциал свободноживущих нематод *Panagrolaimus* sp. (штамм NFS-24-5), как живого корма для ранних стадий развития рыб и креветок в морской аквакультуре. Одной из необходимых предпосылок коммерческого использования является возможность массового производства корма при низких расходах. Целью настоящего исследования было выяснить возможность повышения выхода нематод в моноксенных жидких культурах на клетках дрожжей *Saccharomyces cerevisiae* в колбах и биореакторах. Наблюдения за культивированием в колбах на протяжении 16 дней дали противоречивые результаты. В лабораторных реакторах при условиях постоянного добавления субстрата (fed-batch process) выход существенно снижался. Максимальный выход в больших биореакторах был достигнут при плотности нематод 270×10^3 ml^{-1} для обычных условий культивирования и плотности 153×10^3 nematodes ml^{-1} в случае постоянного добавления субстрата (fed-batch). При культивировании в колбах наблюдалась 8-дневная пауза (lag phase) до начала размножения нематод. Этот период сокращался до 4 дней при культивировании в биореакторах.