Biotechnology and Bioprocess Engineering 22: 319-326 (2017)
DOI 10.1007/s12257-016-0349-4

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

High Concentrations of Biotechnologically Produced Astaxanthin by lowering pH in a *Phaffia rhodozyma* Bioprocess

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Received: 24 May 2016 / Revised: 18 November 2016 / Accepted: 9 April 2017

Abstract Astaxanthin additions to animal diets predominantly serve as colorization aid to satisfy consumer expectations and desire for a consistent product with familiar coloration, *e.g*. the characteristic pink colorization of the flesh of species being produced by aquaculture. The heterobasidiomycetous yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) can be used as natural feed source of astaxanthin. However, currently, the majority of astaxanthin used for the feed market is produced by chemical synthesis. We present a further step in direction of a competitive production of natural astaxanthin in an optimized bioprocess with non-genetically modified *Phaffia rhodozyma*. After medium optimization AXJ-20, a mutant strain of *P. rhodozyma* wild-type strain ATCC 96594, was able to grow to a cell dry weight concentration of over 114 g per kg of culture broth in a fed-batch process. In this bioprocess, where pH was lowered from 5.5 to 3.5 during the maturation phase, AXJ-20 produced the highest value reported for astaxanthin production with *P. rhodozyma* up to now: 0.7 g astaxanthin per kg of culture broth with a space-time-yield of 3.3 mg astaxanthin per kg of culture broth per hour. Lowering the pH during the bioprocess and increasing trace element and vitamin concentrations prevented loss of cell dry weight concentration in the maturation phase and proved to be critical for astaxanthin concentration and purity.

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Keywords: natural astaxanthin, *Phaffia rhodozyma*, *Xanthophyllomyces dendrorhous*, fed-batch bioprocess

1. Introduction

The Food and Agriculture Organization of the United Nations (FAO) reports a steadily increasing importance of aquaculture in total fish supply. In 2012 the global production of farmed fish was 66.6 million tonnes which equals 42.2 percent of the total 158 million tonnes of fish produced by capture fisheries and a market value of US$ 137.7 billion [1]. The global farming of atlantic salmon (*Salmo salar*) produced 2.07 million tonnes in 2012. Since atlantic salmon, rainbow trout and other salmonoids are unable to synthesize carotenoids *de novo* and rely on absorption of carotenoids from their feed sources the common practice in aquafarming is the addition of carotenoids, mostly astaxanthin, to the feed diet to influence pigmentation [2]. Considering the aforementioned numbers on global salmon aquaculture it is not surprising that astaxanthin is the second most important carotenoid with growing global market value [3]. Besides fish farming it finds its application also in the pharmaceutical and food industries [4].

Phaffia rhodozyma, a heterobasidiomycetous yeast, was first isolated from its natural habitat, the wounds of birch trees in colder mountainous regions, by Herman Phaff in the late 1960s. Later, it was taxonomically classified as the new genus *Phaffia* represented by a single species, *Phaffia rhodozyma* [5,6]. In 1995 the teleomorphic state of *P. rhodozyma* was identified and labeled *Xanthophyllomyces dendrorhous* [7]. The reddish color of *P. rhodozyma* colonies derives from the accumulation of xanthophylls (oxygenated carotenoids). Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) represents the majority of 83 ~ 87% of total carotenoids, followed by phoenicoxanthin (5 ~ 7%), 3-

hydroxyechinenone (3 ~ 4%), echinenone (2 ~ 4%) and β-carotene (2 ~ 2.5%) [8]. Xanthophylls serve as antioxidants and quench reactive oxygen species to protect *P. rhodozyma* from damage caused by oxidative stress [9,10]. *P. rhodozyma* has potential commercial value as a source of natural astaxanthin, however the high cost of production limits the use of *P. rhodozyma* [11]. Commercially, *P. rhodozyma* cells are used as components of feed products containing naturally sourced astaxanthin for the aquaculture market, *e.g.* Aquasta®, produced by Naturxan, a joint venture between Archer Daniels Midland Company and Igene Biotechnology, Inc. [12]. Different effects of *P. rhodozyma* based feeds compared to feeds containing synthetic astaxanthin on pigmentation of aquaculture salmonids have been described. While in one study advantageous effects of *P. rhodozyma* based astaxanthin feeds were described [13], another study reported astaxanthin concentration in examined fish was not significantly different between fish fed a *P. rhodozyma* and fish fed a synthetic astaxanthin diet [14].

A comprehensive review on research activities that were realized in the past in order to increase the astaxanthin yield and to lower production costs of *Phaffia* bioprocesses was presented by Schmidt *et al.* [12]. These research activities mainly involved random mutagenesis of *P. rhodozyma* wild type strains and development of suitable bioprocesses. A state-of-the-art industrial bioprocess for the *de novo* production of astaxanthin with *P. rhodozyma* is divided in two phases: a growth phase and maturation phase [15-17]. During the batch phase of the bioprocess, by providing a low C/N ratio (ratio of carbon source to nitrogen source concentration), the cells initially show rapid growth. Growth gradually slows down as the culture approaches high cell concentrations. During this phase, astaxanthin formation is slower than the growth rate of the cells. Subsequently, during the maturation phase, astaxanthin formation ultimately exceeds the cell growth rate. More detailed information on biotechnological astaxanthin production with *Phaffia rhodozyma* or other microbial and herbal sources can be found in several comprehensive reviews [18,19,12].

Current biotechnological production of natural astaxanthin is not cost competitive with the chemical synthesis of this compound. This paper presents a further step towards a cost effective production of natural (3*R*, 3′*R*)-astaxanthin accumulating 0.7 g astaxanthin per kg of fermentation broth utilizing a mutant of *P. rhodozyma* in a bioprocess with an optimized fermentation protocol.

2. Materials and Methods

2.1. Strains

Phaffia rhodozyma strain AXJ-20 was used for astaxanthin production in bioreactor experiments. Strain AXJ-20 is a mutant strain derived from *Phaffia rhodozyma* wild-type strain ATCC 96594 by several rounds of random mutagenesis by Hoshino *et al.* [17].

2.2. Media

Two synthetic media were used for comparing astaxanthin production during fermentation, Seed medium and main medium. The seed medium is a slight variation of a previously published medium [20]. In detail, seed medium was composed of (in g/L) 30 glucose; 4.83 NH_4Cl; 1 KH_2PO_4; 0.88 $MgSO_4 \cdot 7H_2O$; 0.06 NaCl; 0.2 $CaCl \cdot 2H_2O$; 20 potassium phthalate; 0.3 mL/L of trace element solution; 1.5 mL/L of vitamin solution. Trace element solution consisted of 100 mL/L 2 M H_2SO_4 and (in g/L) 50 citric acid; 90 $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$; 16.7 $ZnSO_4 \cdot 7H_2O$; 2.5 $CuSO_4 \cdot 5H_2O$; 1.6 $MnSO_4 \cdot 1H_2O$; 2 H_3BO_3; 2 $Na_2MoO_4 \cdot 2H_2O$; 0.5 KI. Vitamin solution was composed of 17.5 mL/L 2 M H_2SO_4 and (in g/L) 40 myo-inositol; 2 vitamin B3; 2 vitamin B5; 2 vitamin B1; 0.2 vitamin B6; 0.032 vitamin B7; 1.2 *p*-aminobenzoic acid. The pH was adjusted to 5.5 with 5 M NaOH.

The main medium was used as main cultivation medium during the strain comparison in bioreactors. The composition of main medium was as follows (in g/L): 31.5 glucose; 2.45 $(NH_4)_2SO_4$; 14.25 KH_2PO_4; 2.1 $MgSO_4 \cdot 7H_2O$; 0.865 $CaCl \cdot 2H_2O$; 0.28 $FeSO_4 \cdot 7H_2O$; 4.2 mL/L trace element solution; 9.35 mL/L vitamin solution. Trace element solution consisted of 100 mL/L 2 M H_2SO_4 and (in g/L) 50 citric acid; 16.7 $ZnSO_4 \cdot 7H_2O$; 2.5 $CuSO_4 \cdot 5H_2O$; 2 $MnSO_4$; 2 H_3BO_3; 2 $Na_2MoO_4 \cdot 2H_2O$; 0.5 KI. Vitamin solution was composed of 32.5 mL/L 4 N H_2SO_4 and (in g/L) 2 vitamin B3; 3 vitamin B5; 2 vitamin B1; 0.2 vitamin B6; 0.12 vitamin B7; 1.2 *p*-aminobenzoic acid. The pH was adjusted to 5.5 with 5 M NaOH. For some bioreactor experiments the concentration of trace element and vitamin solution was increased to 8.4 and 18.8 mL/L, respectively.

2.3. Seed train

The seed train comprised two consecutive preculture steps before inoculation of the main culture. For preparation of the first preculture 1 mL of cryo culture of AXJ-20 was used to inoculate 20 mL of seed medium. The culture was incubated for 4 days at 21°C at 200 rpm in 100 mL shaking flasks on a rotary shaker (Minitron; Infors; Switzerland). For the second preculture step a fraction of the first preculture was used to inoculate another shaking flask with seed medium at 6.25% (v/v). The second preculture was incubated for 2 days under the same conditions as the first preculture. The criterion for transfer of inoculums was incubation time. In order to start the fermentation process in bioreactors the main culture was inoculated with a share

of the second preculture accounting for 10% (v/v) of starting fermentation volume.

2.4. Main culture

The main culture was incubated in shaking flasks or in lab-scale bioreactors. For initial experiments on the effect of a pH-shift during maturation phase 500 mL baffled shaking flasks were inoculated from the seed train to give a starting culture volume of 110 mL. The shaking flasks were incubated at 21°C and 180 rpm on a rotary shaker (Minitron; Infors; Bottmingen; Switzerland) for 111 h. Measurements of pH were conducted every 4 ~ 12 h with a pH-electrode (InLab Micro; Mettler Toledo; Giessen; Germany) and if necessary pH was adjusted by addition of 27% (v/v) NH_4OH. Glucose concentration was diagnosed in supernatant of centrifuged samples with an automated enzymatic glucose assay in an YSI 2700 glucose analyzer (YSI, Yellow Springs, USA). When necessary, glucose was added from a 500 g/L stock solution. Cell dry weight concentrations were determined by first creating a calibration curve relating the optical density at 600 nm with cell dry weight concentration. Cell dry weight concentration was measured in triplicate from 10 mL samples of *Phaffia* cultures with a MA 100 moisture meter (Sartorius, Goettingen, Germany). During the process optical density was measured spectrophotometrically and cell dry weight concentration was calculated from the calibration curve. After 111 h the pH during maturation phase was changed to 3.5 and 5.5, respectively, by addition of 20 g/L potassium phthalate and adjustment with 5 M NaOH. To set to pH 7.5, 20 g/L Tris were added and 2 M H_2SO_4 was used for final adjustment. Foam formation was suppressed by the addition of anti-foam. The experiments were conducted in duplicate.

2.5. Bioreactor experiments

Bioreactor experiments were conducted in stirred bioreactors (Biostat C-DCU; B. Braun Biotech International GmbH; Melsungen, Germany). Bioreactors possessed a total liquid capacity of 20 L. The starting mass of the fermentation broth was 6 kg. The pH was controlled and automatically adjusted to 5.5 during the fermentation process by addition of gaseous ammonia. The temperature was kept at 21°C. The vessels were aerated with pressurized air with a flow rate of 6 L/min. The dissolved oxygen (DO) was controlled by stirrer speed and kept at 40% once the oxygen consumption of the culture increased. Stirrer speed was varied continuously between 300 and 1,000 rpm to maintain the DO setpoint. Stirrers were equipped with two 6-bladed impellers. Foam formation was suppressed by a conductivity sensor controlled, automated addition of non-autoclaved anti foam (Industrol, BASF, Ludwigshafen). After consumption of the initial glucose during the batch phase of the bioprocess, the fed-batch was started by activating a glucose feed with a 600 g/kg glucose solution and a continuous flow rate. During the fermentation process the continuous feed rate was adjusted in order to keep glucose concentration in the medium at a low concentration, preferably below 5 g/L. Glucose concentration was diagnosed in supernatant of centrifuged samples with an automated enzymatic glucose assay in a Biosen 5130 AutoCal (EKF-diagnostic GmbH, Magdeburg, Germany). The total process time was 216 h.

Cell dry weight concentrations were determined in duplicate by drying a sample of approximately 10 g culture broth at 105°C until constant weight was recorded.

During maturation phase pH was changed by stopping automatic addition of gaseous ammonia until a pH of 3.5 was reached, which took approximately 3 h. Reactor experiments were performed in duplicate.

2.6. Carotenoid extraction and quantification

In shaking flask experiments samples of 1 mL were withdrawn from the culture. A variation of an existing protocol for carotenoid extraction and subsequent HPLC analysis was used [21]. In short, *Phaffia* cells were centrifuged at 14,000 g for 2 min and the cell pellet was dissolved in 1 mL DMSO and heated to 55°C for 10 min. The solution was first extracted with 40 mL diethyl ether then with 40 mL of a 1:1 mixture of acetone and 10% (v/v) diethyl ether in petrol ether. This procedure was repeated until the cell pellet was white. The organic phases were collected, washed with distilled water and subsequently dried over a stream of N_2. For a more detailed procedure description please refer to Visser *et al.* [21]. The carotenoid extract was dissolved in acetone and analyzed by HPLC.

HPLC analysis was performed on a Shimadzu (Shimadzu Deutschland, Duisburg, Germany) HPLC system with a SPD M20A diode array and a CTO 20AC column oven. A 25 cm Alltima C18 reversed-phase column (Grace Alltech, Worms, Germany) with a particle size of 5 μm was used for chromatographic separation. During HPLC analysis the column was kept at a temperature of 32°C. The mobile phase consisted of acetonitrile, methanol and propanol (55:30:15, by volume). The flow rate was 1 mL/min. Carotenoids were detected by diode array detector at 460 nm. Astaxanthin concentrations were calculated by relating peak areas to a calibration curve derived from standard solutions of astaxanthin in acetone. The average purity of astaxanthin in the samples was calculated by relating astaxanthin peak areas to the combined peak areas of all other peaks in sample chromatograms. $Y_{P/X}$ was calculated by dividing the mass of astaxanthin measured in 1 mL sample with the cell mass determined in 1 mL sample.

In bioreactor experiments an aliquot of fermentation

broth (approximately 500 mg, exact weight recorded) was mixed with 60 mL DMSO, 0.5 mL 25% NH_4OH and 0.5 g ascorbic acid as antioxidant and heated to 50°C for 30 min in an ultrasonic bath. Subsequently, the solution was filled up to a volume of 100 mL with ethanol and mixed. A volume of 1 mL of the solution was combined with 50 mg NaCl, 15 mL water, 10 mL ethanol and 5 mL of a 4: 1 mixture of *n*-hexane and dichloromethane, mixed for 1 min and centrifuged. An aliquot of the top *n*-hexane/ dichloromethane phase was analyzed by HPLC.

HPLC analysis was performed on an Agilent HPLC system with a binary pump, a UV detector, column oven and an autosampler with chilling function (all 1100 series, Agilent Deutschland, Waldbronn, Germany). A 125 mm LiChrosorb Si60 column with a particle size of 5 µm was used for chromatographic separation. During HPLC analysis the column was kept at a temperature of 20°C. The mobile phase consisted of *n*-heptane and acetone (88:12, by volume). The flow rate was 1.2 mL/min. Carotenoids were detected by UV-detector at 470 nm. Astaxanthin concentrations were calculated by relating peak areas to a calibration curve derived from standard solutions of astaxanthin in chloroform and *n*-heptane (9:1 by volume). The average purity of astaxanthin in the samples was calculated by relating astaxanthin peak areas to the combined peak areas of all other peaks in sample chromatograms. $Y_{P/X}$ was calculated by dividing the determined concentration of astaxanthin by the determined cell dry weight concentration.

3. Results and Discussion

Table 1 summarizes the accumulated data of the shaking flask and bioreactor experiments and compares important process values to the so far, to the best of our knowledge, best performing lab-scale bioreactor processes for the astaxanthin production with *Phaffia rhodozyma* [15,22].

3.1. Shaking flask experiments

Literature reports suggested a positive effect of pH changes during the maturation phase of the bioprocess [23-25]. Shaking flask cultivations should reveal if this effect could also be shown with AXJ-20. Indeed, lowering of the pH to 3.5 during maturation phase (111 h after inoculation) resulted in significantly increased astaxanthin yield per gram of biomass formed ($Y_{P/X}$, Fig. 1, Table 1). In contrast,

Table 1. Summary and comparison of bioprocess results for the astaxanthin production with *Phaffia rhodozyma* AXJ-20 from this work and the so far best performing lab-scale *Phaffia* bioprocesses described in literature

	Strain	pH during maturation phase	X_{final} (g/L) (total process time (h))	X_{final}[c] (g/kg) (total process time (h))	X_{max} (g/L)	X_{max}[c] (g/kg)	$Y_{P/X}$ (mg/g)	c_{Axn} (mg/L)	c_{Axn}[c] (mg/kg)	Astaxanthin purity	V (L)	m (kg)	STY (mg/L h)	STY[c] (mg/kg h)	Reference
Shaking flask experiments	AXJ-20	3.5	14.0 ± 1.6 (232)		15.2 ± 1.5		5.1 ± 0.2	71.1 ± 4.9		nd	nd		0.3 ± 0.02		This work
	AXJ-20	5.5	21.0 ± 5.9 (232)		21.0 ± 5.9		4.4 ± 0.2	92.2 ± 30		nd	nd		0.4 ± 0.13		This work
	AXJ-20	7.5	22.1 ± 0.4 (232)		22.1 ± 0.4		3.9 ± 0.4	85.4 ± 10		nd	nd		0.4 ± 0.04		This work
Bioreactor experiments	AXJ-20	5.5		54.0 ± 2.7 (216)		103.6 ± 2.0	7.35 ± 0.48		396.5 ± 6.4	60.0 ± 0.2		10.2		1.8 ± 0.0	This work
	AXJ-20[b]	5.5		80.2 ± 2.5 (216)		106.2 ± 1.6	6.89 ± 0.50		552.5 ± 57.3	59.6 ± 1.4		10.5		2.7 ± 0.3	This work
	AXJ-20[b]	3.5		114.3 ± 3.1 (216)		116.3 ± 3.3	6.17 ± 0.30		706.0 ± 53.7	69.8 ± 1.6		10.0		3.3 ± 0.2	This work
	AXJ-20 YB[d]	5.5	55 (216)		84		9.7	531		63.8	0.9		2.5		[22]
	UBV-AX3	5.5	86 (115)		-		6.7	573[a]		-	-		5.0[a]		[15]

c_{Axn} final astaxanthin concentration per liter of fermentation volume; m final fermentation mass; V final fermentation volume; STY space time yield; X_{final} final cell dry weight concentration; X_{max} maximum cell dry weight concentration; $Y_{P/X}$ astaxanthin yield per gram of cell dry weight.
[a]Values calculated from given data.
[b]Bioreactor experiments were conducted with increased vitamin and trace element concentration.
[c]Values are related to mass of fermentation broth.
[d]genetically modified *Phaffia rhodozyma* strain.

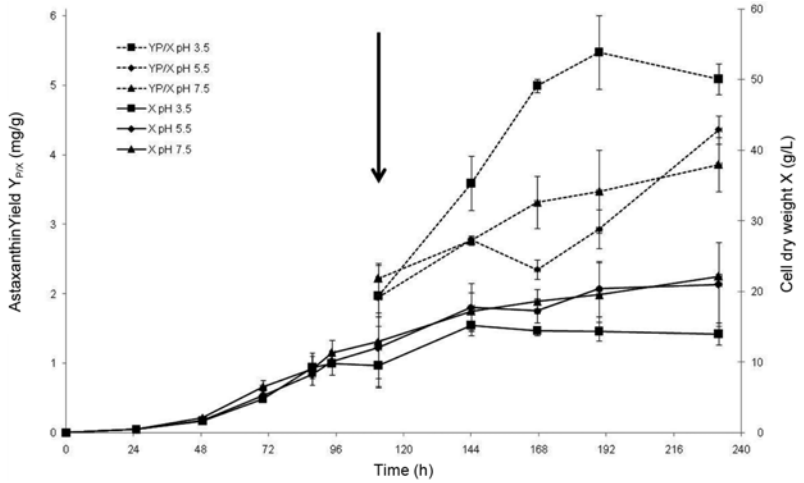

Fig. 1. Astaxanthin yield per gram of cell dry weight ($Y_{P/X}$; mg/g) and cell dry weight concentration (X; g/L) of *Phaffia rhodozyma* AXJ-20 incubated at different pH values during maturation phase. Cells were incubated in baffled shaking flasks at 21°C, pH 5.5. The arrow indicates the time point of pH change, 111 h after inoculation. Experiments were conducted in duplicate.

Phaffia cells incubated at pH 5.5 and 7.5, respectively, produced lower astaxanthin yields per gram of cell dry weight during shake flask experiments. Regardless of pH during incubation astaxanthin yields per gram of cell dry weight ($Y_{P/X}$) increase at a higher rate than the corresponding growth curves during the shake flask experiments. This illustrates the production of astaxanthin after reduction of cell growth, a characteristic of the maturation phase, where astaxanthin accumulation is not coupled with cell growth. The development of cell dry weight concentrations during the growth phase of the bioprocess was comparable between the respective shaking flask experiments at pH 5.5 and 7.5. However, lowering the pH to 3.5 had a slightly negative effect on cell growth (Fig. 1, Table 1), *i.e.* cell dry weight concentration was lower at the end of incubation when cells were grown at pH 3.5. As a consequence of the decreased cell dry weight concentration the final astaxanthin concentration per volume of fermentation volume (c_{Axn}) was lower for the shaking flask experiment with pH-switch to 3.5 (Table 1). In contrast to bioreactor experiments, cell dry weight concentration in shake flask experiments increased over the whole experiment time of 216 h.

3.2. Bioreactor experiments

Transferring the results from shaking flasks experiments, the effect of different pH values during the maturation phase was compared in bioreactors. In addition the impact of increased concentrations of trace elements and vitamins

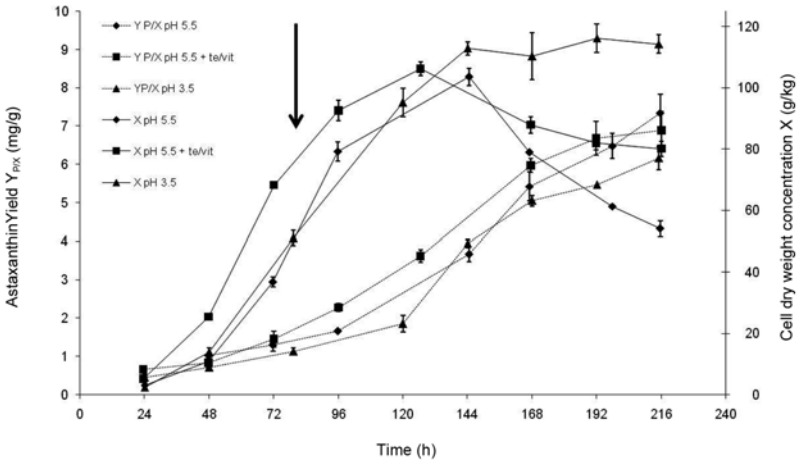

Fig. 2. Astaxanthin yield per gram of cell dry weight ($Y_{P/X}$; mg/g) and cell dry weight concentration (X; g/kg) in a bioprocess with *Phaffia rhodozyma* AXJ-20. A comparison of bioreactor experiments with regular and increased trace element and vitamin concentration (labeled +te/vit) and pH-shift are shown. The arrow indicates the time point of pH-shift from pH 5.5 to 3.5 at 85 h after inoculation. Cultivation was conducted in bioreactors for 216 h in synthetic medium and 21°C. Experiments were performed in duplicate.

was investigated. Results concerning cell mass formation and $Y_{P/X}$ are presented in Fig. 2. Corresponding to the shaking flask experiments, the growth rates during early phases of the growth phase of the bioreactor process were comparable between the respective experiments (Fig. 2). AXJ-20 typically grew with a maximum growth rate of 0.06 ~ 0.07/h. One significant difference in growth development between the AXJ-20 bioprocess was detected during the maturation phase. After approximately 120 h cell dry weight concentration of bioreactor experiments with a constant pH of 5.5 started to decrease. A severe loss of almost 50% of cell dry weight concentration was monitored in bioprocesses with regular trace element and vitamin concentration. Doubling the trace element and vitamin concentration of the main medium somehow lessened this effect but still a loss of cell dry weight concentration of over 20% over a period of 96 h was measured (Fig. 2). The decrease of cell dry weight concentration was accompanied by a color change of the culture's supernatant when cells settled, pointing to cell lysis as possible cause of the reduction of cell dry weight concentration. Also, doubling the concentration of the trace elements and vitamins in the main medium slightly improved astaxanthin yield (Table 1). In contrast, the cell dry weight concentration of the bioprocesses with pH-shift from 5.5 to 3.5 stayed constant at around 114 g per kg of fermentation broth during the stationary phase. At the end of the bioprocess the cell dry weight concentration was almost 30% higher in the bioprocess with pH-shift to 3.5 (Table 1). Apparently, a pH shift to pH 3.5 did not impair cell growth of AXJ-20. Optimal pH values for cell growth and astaxanthin production have been described for different *P. rhodozyma* strains to lie between pH 5 and 6 [12]. However, compared to the wild-type strain ATCC 96594, reduced growth rates were found for *Phaffia rhodozyma* strain AXJ-20 as already described by Gassel *et al.* [22]. Decrease in biomass yields, growth rates and increased lag phases were often observed with *Phaffia* strains generated by random mutagenesis [26-28,15] reflecting the changes in mutant cell metabolism which apparently deviates more resources to carotenogenesis than to cell biomass generation compared to the wild-type. Decreasing cell dry weight concentrations during maturation phase were detected both, in earlier studies of *P. rhodozyma* [29-35] and with randomly mutagenized strain AXJ-20 [22,36]. From data published by Gassel *et al.* [22] it was not clear if decreasing cell dry weight concentrations were a prominent characteristic in AXJ-20 or if process parameters caused this effect. This study revealed the pH and, to some extent, the concentration of trace salts and vitamins as critical parameters for maintenance of cell density during late phases of the bioprocess. The prevention of the loss of astaxanthin producing cells during stationary phase of the bioprocess proved to be imperative to further enhancements in astaxanthin concentration and space-time yield. A pH of 3.5 enabled AXJ-20 to maintain astaxanthin production without loss of cell mass, however, pH 3.5 was suboptimal for the rate of astaxanthin accumulation as shown by decreased $Y_{P/X}$ in comparison to processes at pH 5.5 (Table 1). At pH 5.5 the benefit of increased astaxanthin production rates could not quench the loss of astaxanthin producing cell mass, resulting in a stagnation of c_{Axn} in late phases of the bioprocess, regardless of vitamin or trace element concentration (Fig. 3).

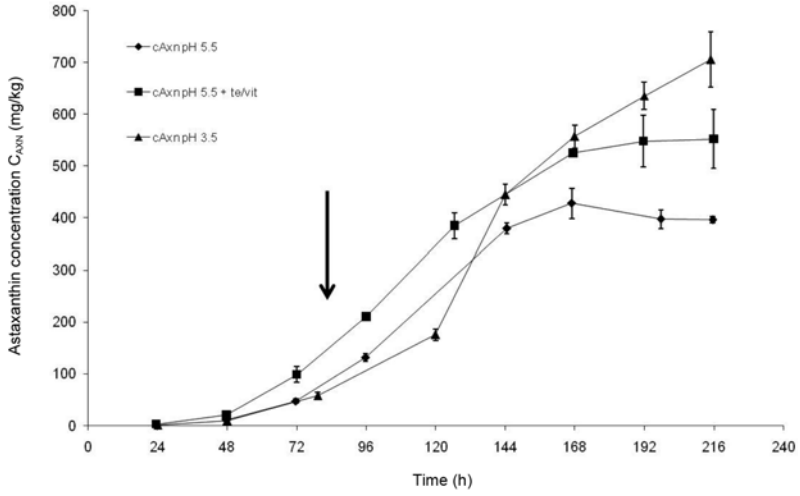

Fig. 3. Astaxanthin concentration in relation to total mass of culture broth (c_{Axn}; mg/kg) of *Phaffia rhodozyma* AXJ-20 during the cultivation in bioreactors. A comparison of bioreactor experiments with regular and increased trace element and vitamin concentration (labeled +te/vit) and pH-shift are shown. Arrow indicates the time point of pH-shift from pH 5.5 to 3.5 at 85 h after inoculation. Experiments were conducted in duplicate.

Astaxanthin purity significantly increased from approximately 60% to approximately 70% when pH was lowered to 3.5 (Table 1). The major byproducts measured were β-carotene, phoenicoxanthin (adonirubin), echinenone, 3-hydroxyechinenone. A stepwise improvement of the concentration of astaxanthin per kg of fermentation broth (c_{Axn}; mg/kg) was achieved by the changes made to the original bioprocess (Fig. 3). Doubling the concentration of trace salts and vitamins improved final c_{Axn} by approximately 30%. By additionally lowering pH to 3.5 a further increase of c_{Axn} of approximately 12% compared to the bioprocess without pH-shift was accomplished (Table 1). Lowering the pH to 3.5 caused a small break-in in c_{Axn} development between 85 and 120 h after inoculation which was, however, followed by an increase in space-time yield (Fig. 2). Astaxanthin accumulation was indeed slower at pH 3.5, however, the astaxanthin fraction of total carotenoids, *i.e.* astaxanthin purity, was increased. Astaxanthin synthase (asy) catalyzes the final step of astaxanthin biosynthesis in *P. rhodozyma*. Asy oxidizes β-carotene to astaxanthin [37] and seems to be the limiting step in astaxanthin synthesis in AXJ-20 at pH 5.5. Increased concentrations of β-carotene, caused by random mutations in AXJ-20, obviously overloads the oxidative capacity of asy resulting in larger fractions of byproducts derived from incomplete oxidation of β-carotene in comparison to the wild-type where astaxanthin represents the majority with 83 ~ 87% of total carotenoids [8]. As a hypothesis a pH value of 3.5 may lower overall metabolic flow thereby decreasing the concentration of asy substrates and resulting in increased astaxanthin purity. At pH 3.5, the maintenance of high cell concentrations compensate for the effect of slower astaxanthin production rate. Considering the trend of c_{Axn} for the process with pH 3.5 (Fig. 3), an extension of overall process time most likely could increase astaxanthin concentration, however, without significant effects on space time yield. A compromise between maintenance of cell mass and rate of astaxanthin biosynthesis may be found by further optimizing the pH value through adjustments between 3.5 and 5.5. Also, an increase in oxygen availability might further improve astaxanthin purity since oxygen plays a pivotal role in astaxanthin biosynthesis [38,34]. Concerning final cell dry weight concentration and astaxanthin concentration the lab-scale bioprocess with pH-shift to 3.5 outplayed all so far published data for astaxanthin production with *P. rhodozyma* (Table 1).

4. Conclusion

By reduction of pH during maturation phase the astaxanthin concentration produced by AXJ-20 raised to 0.7 g astaxanthin per kg of fermentation broth, the highest value reported for biotechnological production of astaxanthin by *P. rhodozyma* so far. Applying the knowledge gathered in this work to bioprocesses utilizing genetically enhanced *Phaffia* strains, especially recombinant *Phaffia* strains with additional copies of astaxanthin synthase, may result in increased intracellular astaxanthin concentrations and purities. This could raise astaxanthin concentrations to over 1 g/L in the future. This work represents a further step to commercial application of biotechnological production of astaxanthin by *Phaffia rhodozyma*.

Acknowledgement

This work was funded by the German Federal Ministry of Education and Research (BMBF) (FKZ 0315327).

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