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Definition of culture conditions for *Arxula adeninivorans*, a rational basis for studying heterologous gene expression in this dimorphic yeast

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Abstract The yeast *Arxula adeninivorans* is considered to be a promising producer of recombinant proteins. However, growth characteristics are poorly investigated and no industrial process has been established yet. Though of vital interest for strain screening and production processes, rationally defined culture conditions remain to be developed. A cultivation system was evolved based on targeted sampling and mathematical analysis of rationally designed small-scale cultivations in shake flasks. The oxygen and carbon dioxide transfer rates were analyzed as conclusive online parameters. Oxygen limitation extended cultivation and led to ethanol formation in cultures supplied with glucose. Cultures were inhibited at pH-values below 2.8. The phosphorus demand was determined as 1.55 g phosphorus per 100 g cell dry weight. Synthetic SYN6 medium with 20 g glucose l^{-1} was optimized for cultivation in shake flasks by buffering at pH 6.4 with 140 mmol MES l^{-1}. Optimized SYN6 medium and operating conditions provided non-limited cultivations without by-product formation. A maximal specific growth rate of 0.32 h^{-1} and short fermentations of 15 h were achieved. A pH optimum curve was derived from the oxygen transfer rates of differently buffered cultures, showing maximal growth between pH 2.8 and 6.5. Furthermore, it was shown that the applied medium and cultivation conditions were also suitable for non-limiting growth and product formation of a genetically modified *A. adeninivorans* strain expressing a heterologous phytase.

Keywords *Arxula adeninivorans* · pH optimum · Screening conditions · Medium development

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Background

Yeasts are well-established producers of recombinant proteins [15, 18]. The range of biotechnologically applied species includes an increasing number of non-*Saccharomyces* species. Some of them, like *Hansenula polymorpha*, are already recognized producers of pharmaceutical proteins [8, 48], whereas others have yet to be established. One species of biotechnological potential is *Arxula adeninivorans.* It is a non-pathogenic organism and harbors neither pyrogenic compounds nor viral inclusions [8, 17]. A transformation system exists and various strong constitutive and inducible promoters are available. Heterologous genes are mitotically stable integrated into the genome, supporting reproducible and stable fermentation processes. Secretion of proteins is provided, easing product recovery. Several auxotrophic host strains are at hand, overcoming the requirement of antibiotic compounds for the generation of recombinant strains. A broad range of heterologous genes have already been expressed in *A. adeninivorans*, contributing to its applicability as a producer of recombinant

proteins [8]. The great diversity of carbon and nitrogen (N) sources utilized by *A. adeninivorans* [35] as well as nutrient-dependent inducible promoters provide options for substrate-based fermentation and expression control. Its osmotolerance enables the use of pH buffers in high concentrations in non-titrated shaking cultures and the employment of highly concentrated media for high-cell-density fermentations [28, 29, 52]. *A. adeninivorans* can grow at temperatures up to 48 °C, thereby enabling fermentation at a broad range of temperatures [50]. Whereas *A. adeninivorans* has been cultivated at laboratory scale (1.25-l fermentor) to produce tannase [6], most of the research on *A. adeninivorans* focuses on molecular biological aspects of gene expression [7, 8] that is based on shake-flask cultures (e.g., [45]).

Fermentation studies at larger scale are limited [6, 28, 29, 36] and fermentation on an industrial scale has not yet been carried out. So far, studies on growth and cultivation are restricted to aspects of temperature-dependent dimorphism and osmotolerance [43, 50, 52]. Minocha et al. [36] reported about the optimization of acid phosphatase production. Recently, *A. adeninivorans* has been used in an estrogen screen assay [25, 38]. Nevertheless, little is known about growth characteristics and rationally defined conditions for *A. adeninivorans* in liquid cultures. This greatly contrasts with the repeatedly stated large biotechnological interest in *A. adeninivorans* as industrial production platform for recombinant proteins. If growth characteristics and culture conditions are not regarded, strain screening may become misdirected and recombinant protein production may become irreproducible or suboptimal [37, 42, 46, 48].

Wild-type strain *A. adeninivorans* LS3 is considered to provide the most appropriate basis for the study presented here as it serves as parental strain for most of the heterologous genes expressed in recombinant *A. adeninivorans* [8]. Thus, the general growth characteristics of strain LS3 can be assumed to correspond to its recombinant derivatives and basic, non-limiting culture conditions defined for strain LS3 are considered to be widely applicable to recombinant strains. In our study, we focus on the impact of oxygen supply, phosphorus (P) supply, and pH on cultivation, determining online the oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) as well as several offline parameters. Whereas in some cases the nature of the recombinant protein defines the pH value of the applied medium, often pH values best suited for maximal growth of the applied organism are chosen. Thus, a systematic approach based on mathematical correlations between pH and growth was chosen to derive a pH optimum curve for growth of *A. adeninivorans*. Thereby, the study presents a method for efficiently evolving a cultivation system based on intense targeted probing of a limited number of rationally designed experiments at small-scale level.

Materials and methods

Strain

Wild-type strain *Arxula adeninivorans* (*Blastobotrys adeninivorans*) LS3 (PAR-4) was applied. The strain was initially isolated from wood hydrolysates in Siberia (Russia) [19]. Strain LS3 is dimorphic, forming budding cells at temperatures below 42 °C and exhibiting filamentous growth above 42 °C [50].

In addition, *Arxula adeninivorans* G1211 (*aleu2*) was used as a genetically modified strain. It is a derivative of the wild-type strain *A. adeninivorans* LS3 and was transformed and described by Wartmann et al. [51]. It produces a heterologous phytase mutein controlled by the *TEF1* gene promoter [41] and was kindly provided by Prof. Gotthard Kunze from the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany.

General cultivation conditions

The cultures were agitated on a shaking machine (Lab-Shaker, Kühner AG, Switzerland) with an orbital shaking diameter of 50 mm and at a standard shaking frequency of 350 rpm. If not specified otherwise, unbaffled 250-ml Erlenmeyer flasks with cotton plugs and 250-ml measuring flasks of the respiration activity monitoring system (RAMOS) device (see section "OTR and CTR Measurement") with a standard filling volume of 10 ml were used for cultivation. Cultivations were carried out at 30 °C. Cultures in cotton-plugged Erlenmeyer flasks served for the determination of offline parameters and were run simultaneously to the cultivations in measuring flasks.

Precultivation

Yeast-Extract-Peptone-Dextrose (YEPD) plates seeded with *A. adeninivorans* LS3 were incubated 2–4 days at 30 °C and were used for the inoculation of the liquid precultures. The YEPD plates contained per liter 10 g yeast extract (Roth, Germany), 20 g gelatin (Difco, Becton–Dickinson, San Jose, CA, USA), 20 g glucose monohydrate, and 18 g agar. Preculturing was carried out with the medium also applied to the main cultures. The precultivations were conducted in measuring flasks and were monitored by means of the RAMOS device (see section "OTR and CTR measurement"). Thereby, cells could be harvested reproducibly at the late exponential growth phase,

corresponding to a final OTR of about 0.04 mol l^{-1} h^{-1} and a final biomass of about 10 g cell dry weight (CDW) l^{-1}.

Main cultivation

The main cultivations were carried out with media derived from synthetic SYN6 medium [12, 23]. Glycerol of the original SYN6 medium was replaced by glucose as carbon source (this medium variant is denoted as SYN6-PO_4 in the following). SYN6-PO_4 contained per liter (for preparation procedure refer to Hellwig et al. [23]): 20 g glucose, 13.3 g $NH_4H_2PO_4$, 3.3 g KCl, 3 g $MgSO_4{\cdot}7H_2O$, 0.3 g NaCl, 1 g $CaCl_2{\cdot}2H_2O$, 66.7 mg $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, 5.3 mg $CuSO_4{\cdot}5H_2O$, 20 mg $ZnSO_4{\cdot}7H_2O$, 26.7 mg $MnSO_4{\cdot}H_2O$, 0.7 mg $NiSO_4{\cdot}6H_2O$, 0.7 mg $CoCl_2{\cdot}6\ H_2O$, 0.7 mg H_3BO_3, 0.7 mg KI, 0.7 mg $Na_2MoO_4{\cdot}2H_2O$, 66.7 mg ethylenediaminetetraacetate (Titriplex III, Merck, Germany), 0.4 mg D-biotin, (Sigma-Aldrich, St. Louis, MO, USA), 133.3 mg thiamine chloride-hydrochloride (AppliChem, Darmstadt, Germany). SYN6-PO_4 has an initial pH of 4.0 without being titrated. SYN6-PO_4 was varied in two sets of cultivations. In the first set (see sections "pH buffering" and "Systematic approach to characterize growth as a function of pH"), different pH buffer compounds were introduced into SYN6. In these media, 13.3 g $NH_4H_2PO_4$ l^{-1} was replaced by 1 g KH_2PO_4 l^{-1} and 7.66 g $(NH_4)_2SO_4$ l^{-1}, in order to maintain P and N supply. Additionally, either 44.3 g homopiperazine-1,4-bis(2-ethanesulfonic acid) (HOMOPIPES) l^{-1} (Sigma-Aldrich), 27.3 g 2-(*N*-morpholino)ethanesulfonic acid (MES) l^{-1} (Sigma-Aldrich), 18.05 g KH_2PO_4 l^{-1}, or 33.36 g 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) l^{-1} (Sigma-Aldrich) were introduced as pH buffer compounds (equivalent to final molar concentrations of 140 mmol l^{-1}). The resulting media were adjusted to initial pHs as denoted in Table 1. In the second set of cultivations (see section "Phosphorus supply"), the nutritional P demand was analyzed. For this purpose, the phosphate concentration of MES-buffered SYN6 (SYN6-MES, see above) was adjusted to 0.2, 0.45, 1.0, and 2.0 g KH_2PO_4 l^{-1}, respectively, equaling P concentrations of 46, 103, 228, and 456 mg P l^{-1}. The main cultures were inoculated with preculture broth, using one hundredth of the main culture volume.

OTR and CTR measurement

The OTR and CTR were measured online with a respiration activity monitoring system (RAMOS) device made in house [1, 2]. Commercial versions are available at Kühner AG, Switzerland, or HiTec Zang GmbH, Germany. Oxygen partial pressure sensors and differential pressure sensors are used to monitor the oxygen and carbon dioxide partial pressure in the head spaces of eight measuring flasks in parallel. Using these data, the oxygen transfer rate (OTR), the carbon dioxide transfer rate (CTR), and the respiratory quotient (RQ) is calculated. The respective cultivations are conducted in specially designed measuring flasks providing hydrodynamics and gas phase conditions equivalent to those in normal shaken Erlenmeyer flasks with cotton plug [2]. The transfer rates reflect the microbial respiration. This technique has already successfully been applied in several previous studies (e.g., [22, 30, 31, 44, 46, 47]).

Standard analytics

The pH of the culture broths was determined potentiometrically with a pH meter (type 740, Knick, Germany) and a pH electrode (InLab 406, Mettler Toledo, Switzerland). Biomass was determined gravimetrically as CDW. Five milliliters of culture broth was filtered through dried and pre-weighed cellulose acetate filters (pore size 0.2 µm, 11107-47-N, Sartorius, Germany). After resuspension of the filter residue with sodium chloride solution (9 g l^{-1}) and repeated filtration, the filters were dried at 105 °C

Table 1 Specification of the pH bufferings employed in the investigation for the determination of the specific growth rate as a function of pH

Buffer	Initial pH (−)	Dissociation constant pK_a at 30 °C (−)	Buffer concentration (mmol l^{-1})
Phosphate buffer (phosphoric acid/dihydrogen phosphate)	4.0	2.17	120
HOMOPIPES	4.8, 5.2, 6.0	4.67	140
MES	6.1, 6.4	6.04	140
Phosphate buffer (dihydrogen phosphate/monohydrogen phosphate)	7.2	7.19	140
HEPES	7.7	7.41	140

The cultivation series comprised 17 shake-flask cultures in SYN6 derivatives under different pH conditions, buffered as denoted. The cultivations were conducted under non-limiting standard conditions as denoted in the section "Materials and methods". Initial pH pH(0), dissociation constant of the buffer pK_a, and buffer concentration $c_{HA + A^-}$ are input variables for the calculation of pH over fermentation time pH(t). For the dissociation constants pK_a refer to Good et al. [20], and Blanchard [5]

until mass remained constant. Culture broth was analyzed for glucose and ethanol with a high-performance liquid chromatography system (Dionex Softron, Germany). The system was operated with Chromeleon Software (V6.4, Dionex Softron, Germany). An organic acid resin (250 × 8 mm, CS Chromatographie Service, Germany) served as stationary phase. The filtered culture samples (0.22 µm, CM, Qualilab, VWR International, Germany) were diluted 1:2 with 1 mmol sulfuric acid l^{-1}. A total of 20 µl of this dilution was isocratically eluted with 1 mmol sulfuric acid l^{-1} at a flow rate of 0.6 ml min^{-1} and a temperature of 30 °C. Eluents were detected by a refractive index detector (Shodex RI-71, Techlab, Germany).

Measurement of phytase activity

Phytase activity was measured kinetically as described by Stöckmann et al. [46]. One phytase unit (FTU) is defined as the amount of enzyme that releases 1 µmol inorganic phosphate per minute from sodium phytate at 37 °C and a pH value of 5.5.

Results and discussion

Selection of synthetic medium

Synthetic SYN6 medium originally developed for the fermentation of recombinant strains of the yeast *H. polymorpha* was considered to be an ideal basis for the cultivation of *A. adeninivorans* in our study. With 7.66 g $(NH_4)_2SO_4$ l^{-1}, nitrogen is present in abundance in this medium, allowing the production of heterologous proteins without running into a nitrogen limitation. Therefore, the nitrogen source is not discussed further in this study. The standard carbon source glycerol was replaced by glucose. The remaining SYN6 compounds were kept unchanged for the employment in shaking cultures to a maximum possible extent although they are present in relatively high concentrations optimal for high-cell-density fermentations (HCDFs). In this way, media-based inconsistencies between shaken screening cultures and production in HCDFs can be reduced since SYN6 is intended to be also employed in HCDFs of *A. adeninivorans*. Synthetic media are generally a preferred option for the production of recombinant proteins because of their beneficial properties with regard to protein purification, product yield, and process consistency [21]. Additionally, synthetic SYN6 is chemically defined and exhibits defined physicochemical properties, which are prerequisites for the mathematical description of the cultures in our study.

Oxygen supply and operating conditions

Initially, the general characteristics of *A. adeninivorans* cultures were assessed applying different conditions of oxygen supply. Figure 1a, c depict a predominantly oxygen-limited culture of *A. adeninivorans* in SYN6-PO_4. In the beginning, the OTR increased exponentially in accordance with a non-limited exponential growth. Simultaneously, CTR increased corresponding to a respiratory quotient (RQ) of 1.1 (data not

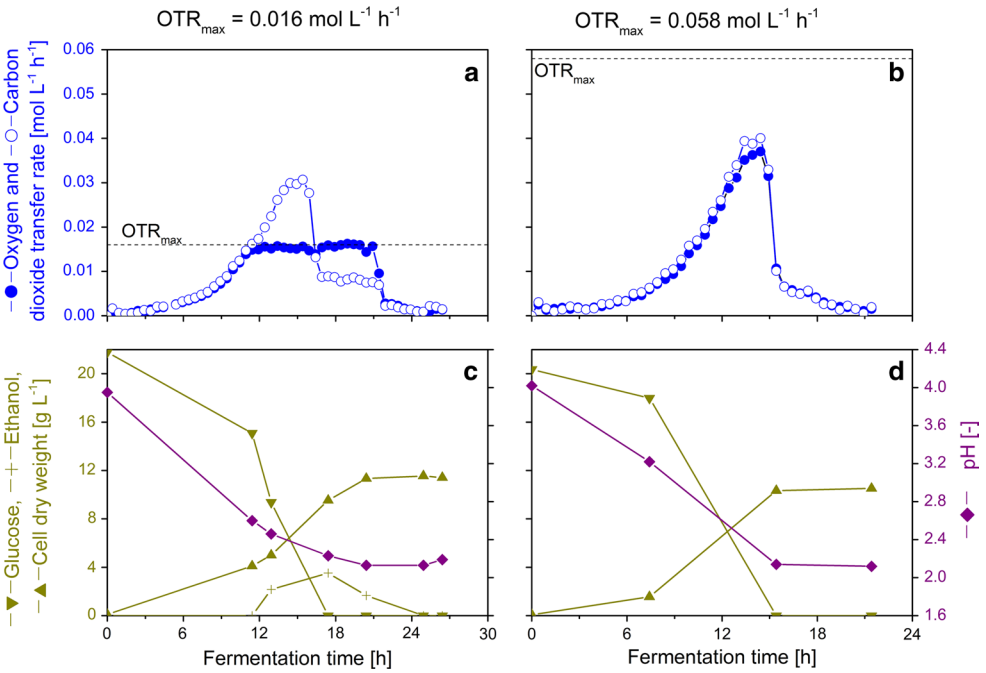

Fig. 1 *A. adeninivorans* LS3 cultures in SYN6-PO_4 under conditions for a predominantly limiting (**a**, **c**) and a non-limiting (**b**, **d**) oxygen supply. Courses of OTR, CTR (**a**, **b**). Courses of glucose, ethanol, cell dry weight, and pH (**c**, **d**). The operating conditions result in maximum oxygen transfer capacities (OTR_{max}) of 0.016 mol l^{-1} h^{-1} (**a**, **c**) and 0.058 mol l^{-1} h^{-1} (**b**, **d**): orbital shaking diameter 50 mm, shaking frequency 350 rpm, shake flask 250 ml, liquid volumes 60 ml (**a**, **c**) and 10 ml (**b**, **d**), temperature 30 °C

shown), indicating the growth-coupled aerobic turnover of glucose. At hour 11, the maximum oxygen transfer capacity (OTR_{max}) of 0.016 mol l^{-1} h^{-1} was reached, constantly limiting the OTR to this level. In contrast, the CTR continuously increased to 0.030 mol l^{-1} h^{-1}, resulting in a maximal RQ of 2.0 which is indicative of a fermentative metabolism. When glucose was depleted at hour 17, CTR declined rapidly. During this first phase, the fermentative formation of 3.5 g ethanol l^{-1} was observed, subsequently followed by ethanol metabolization (Fig. 1c). This was accompanied by a CTR of 0.008 mol l^{-1} h^{-1} below the OTR, corresponding to a RQ of 0.5, which is indicative of the growth-coupled aerobic turnover of the reduced and non-fermentable carbon source ethanol. When ethanol was depleted at hour 22, the transfer rates completely dropped down, indicating the onset of the stationary phase. The consistency of the culture stoichiometry was assessed by a material balance [3, 46]. This involved all substrates and products of the culture described in Fig. 1a, c. Balancing of elemental carbon and oxygen yielded a gap of less than 4 % between substrates and products. This suggested that all significant substrates and products of the culture were included, thereby confirming the conclusiveness of the culture characterization.

As shown in Fig. 1a, c, oxygen limitations extend process times and influence metabolism. Though a common finding, this vital aspect is still often neglected. Ignoring the oxygen supply, productivities may be irreproducible and inconsistent between scales. Strain screening may be misdirected [37, 42, 46]. Special caution is advised if promoters sensitive to carbon sources are employed [7]. In case of an oxygen limitation (Fig. 1a, c), heterologous genes are now additionally expressed under the exposure to ethanol instead exclusively to glucose. These considerations demonstrate the demand for a well-adjusted, non-limiting oxygen supply.

The oxygen supply in shaking cultures is limited by the maximum oxygen transfer capacity OTR_{max}. As long as the OTR stays below the OTR_{max}, a non-limiting oxygen supply is provided. The OTR_{max} of a shaking culture is predominantly a function of the operating conditions and the physicochemical properties of the employed medium. If the OTR_{max} is known for a set of operating conditions, as it is the case in the SYN6-PO_4 culture shown in Fig. 1a, c, OTR_{max} values for a broad range of operating conditions can be calculated according to Maier et al. [33] and Büchs et al. [10], respectively. Following their considerations, operating conditions for an increased OTR_{max} were defined as applied to the SYN6-PO_4 culture described in Fig. 1b, d. For this purpose, the liquid volume was reduced from 60 to 10 ml. Thereby, the OTR_{max} almost quadrupled from 0.016 mol l^{-1} h^{-1} (Fig. 1a) to a non-limiting value of 0.058 mol l^{-1} h^{-1} well above the maximal OTR of the culture (Fig. 1b). Under these conditions, the OTR increased exponentially until hour 11. Subsequently, the OTR curve adopted a sigmoidal shape, reaching a maximum of 0.037 mol l^{-1} h^{-1}. This phenomenon points to a gradually increasing inhibition of the culture [1], and is discussed in the section "pH buffering". The CTR was slightly higher than the OTR and increased at the same time as the OTR, resulting in a RQ between 1.1 and 1.2. This again corresponds to a growth-coupled aerobic turnover of glucose. At hour 15, OTR and CTR dropped down, thereby indicating glucose depletion and the onset of the stationary phase. The culture exhibited a balance gap of 4 % and less with respect to carbon and oxygen. In contrast to the oxygen-limited culture (Fig. 1a, c), no by-products were formed under the conditions for a non-limiting oxygen supply (Fig. 1b, d), which was in agreement with the consistent material balance and the coherent RQ, respectively. While in both SYN6-PO_4 cultures similar biomass yields of ca. 0.52 g CDW g $glucose^{-1}$ were reached irrespective of the OTR_{max}, cultivation time at non-limiting oxygen supply was reduced to two-thirds of the time required for the oxygen-limited cultivation (Fig. 1a–d).

pH buffering

In both SYN6-PO_4 cultures shown in Fig. 1a–d, the pH decreased strongly from initially 4.0 to a low final value of 2.1. Thus, the inhibition indicated by the OTR under the conditions for a non-limiting oxygen supply (Fig. 1b) was most likely due to the strongly acidic final pH conditions. The pH influencing microbial metabolism is a well-known matter frequently discussed (e.g., [13, 21, 32]). In this respect, non-inhibiting and reproducible pH conditions should be adopted for strain screening and production, supporting vital growth and the consistency of cultivation results.

The established pH buffering of SYN6-PO_4 with phosphate is not useful for effective pH stabilization, since its initial pH of 4.0 is outside of the buffer ranges of the phosphate systems, phosphoric acid/dihydrogen phosphate (pK_a = 2.17) and dihydrogen/monohydrogen phosphate (pK_a = 7.2) (see Table 1) [5]. Therefore, MES buffer [20] was introduced into SYN6. At the same time, the phosphate concentration of SYN6-PO_4 was reduced from 120 to 7.35 mmol l^{-1} to ensure the solubility of MES buffer and the P supply of the cultures (for the definition of the cellular P demand refer to section "Phosphorus supply"). A total of 140 mmol MES l^{-1} and an initial pH of 6.4 were found suitable for pH stabilization as deduced from mathematical predictions of pH over fermentation time (refer to section "Systematic approach to characterize growth as a function of pH"). Figure 2a, b depict an *A. adeninivorans* culture in SYN6-MES under conditions for a non-limiting oxygen

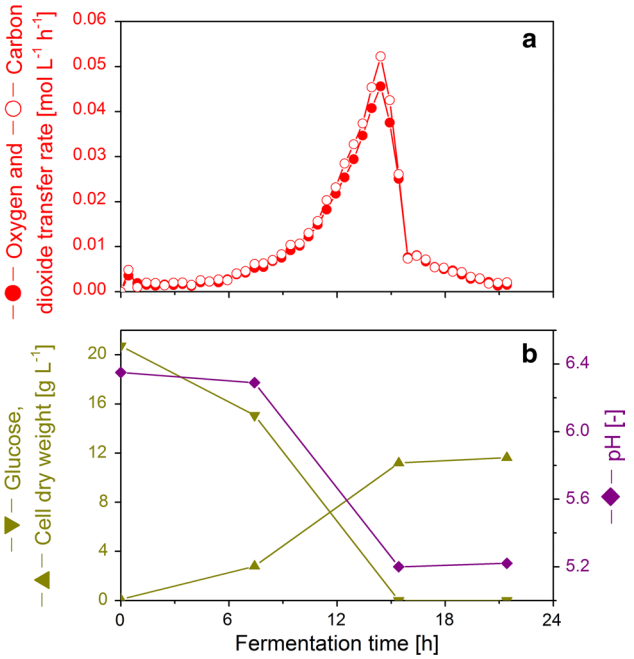

Fig. 2 *A. adeninivorans* LS3 culture in SYN6-MES (140 mmol MES l^{-1}, initial pH 6.4). Courses of OTR, CTR (**a**). Courses of glucose, cell dry weight, and pH (**b**). Operating conditions (for a non-limiting oxygen supply): orbital shaking diameter 50 mm, shaking frequency 350 rpm, shake flask 250 ml, liquid volume 10 ml, temperature 30 °C

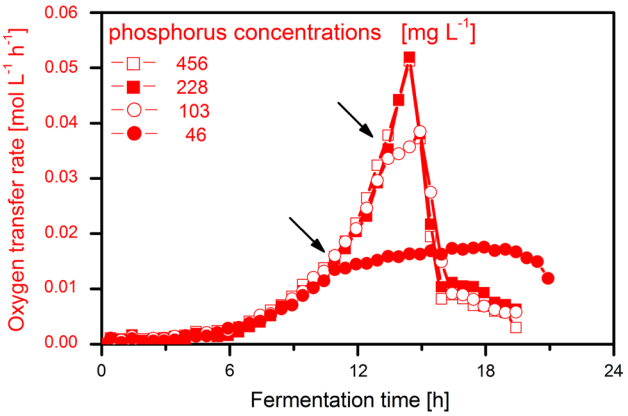

Fig. 3 OTR courses of *A. adeninivorans* LS3 cultures in SYN6-MES under conditions of limiting and excessive phosphorus concentrations. Phosphorus concentrations: 46, 103, 228, and 456 mg l^{-1}. *Arrows* denote the time of phosphorus exhaustion in the phosphorus-limited cultures. Operating conditions (for a non-limiting oxygen supply): orbital shaking diameter 50 mm, shaking frequency 350 rpm, shake flask 250 ml, liquid volume 10 ml, temperature 30 °C

supply. The pH could be successfully stabilized within close limits of initially 6.4 to finally 5.2 (Fig. 2b) compared to the decrease of the pH from 4.0 to 2.1 in the SYN6-PO_4 cultures (Fig. 1c, d). The OTR and CTR increased exponentially with increasing biomass until the end of the cultivation at hour 15, reflecting a continuous maximal specific growth rate of 0.32 h^{-1}. No sigmoidal shape of the OTR curve is recognized, in contrast to the culture shown in Fig. 1b. Thus, pH of the culture was successfully stabilized adopting non-inhibiting values. Similar maximal specific growth rates observed in the oxygen-limited or pH-inhibited SYN6-PO_4 cultures were found to be restricted to the initial phases (Fig. 1a–d).

In fast growing yeast cultures under aerobic conditions such as the SYN6-MES culture (Fig. 2a, b), high glucose uptake rates may lead to the formation of overflow metabolites [16, 46]. Nevertheless, a continuous RQ of 1.1 (data not shown) during growth indicated a stable aerobic metabolism without by-product formation. Furthermore, the material balance of the culture was consistent with a gap of less than 4 % related to carbon and oxygen, which again suggested that no significant by-product formation was present. A biomass yield of 0.56 g CDW g glucose^{-1} was obtained in the SYN6-MES culture compared to 0.52 g CDW g glucose^{-1} in the SYN6-PO_4 cultures (Fig. 1a–d). The high ionic strength of SYN6-MES (SYN6: ca. 0.9 mol l^{-1} [47]) obviously did not affect culturing of this osmotolerant yeast species.

Phosphorus supply

A non-limiting P supply is a decisive factor for culturing since phosphate plays a central role in metabolic control and energy metabolism [49]. P is critical for growth and can also influence gene expression [26, 34]. The reduced P supply of SYN6-MES (see section "pH buffering") has to be non-limiting. Therefore, the theoretical P demand of the cultures was initially approximated based on the elemental composition of yeast. Data on P content of different yeast species under diverse environmental conditions vary greatly [11, 24]. Between 0.4 and 2.0 g P per 100 g CDW are reported in literature [21], e.g., 2.02 g P per 100 g CDW for *S. cerevisiae* by Duboc et al. [14]. Because of this extensive variance, the specific P demand of *A. adeninivorans* had to be determined. For this purpose, *A. adeninivorans* was cultured in SYN6-MES with presumably limiting and excessive P concentrations (Fig. 3). The cultures supplied with 228 and 456 mg P l^{-1} exhibited non-limited OTR courses, and thus were obviously not affected by a P limitation. In contrast, the exponential increase of the OTR of the cultures supplied with 46 and 103 mg P l^{-1} diminished abruptly after 11 and 14 h, respectively, indicative for metabolic changes due to the P exhaustion in the media.

Linear correlations between biomass formation, oxygen and P consumption during non-limited growth can be postulated. Thus, an oxygen-dependent biomass yield Y_{X/O_2}

could be calculated from the total oxygen consumption and the total biomass formation of the non-limited cultures (Fig. 3, cultures supplied with 228, 456 mg P l^{-1}).

The oxygen consumption of the P-limited cultures during non-limited growth until P depletion and the underlying initial P concentrations of the media (Fig. 3, cultures supplied with 46, 103 mg P l^{-1}) served for the determination of the oxygen-dependent P consumptionR_{P/O_2}. The division of $R_{P/O_2}$by Y_{X/O_2} yielded the biomass-dependent P demand, which was calculated as 1.55 g P per 100 g CDW. Corresponding to a final biomass of about 11.7 g CDW l^{-1} in non-limited SYN6-MES cultures (Figs. 2a, b, 3, cultures supplied with 228, 456 mg P l^{-1}) a minimal supply with 181 mg P l^{-1} was found to be essential for non-limiting conditions. Thus, 228 mg P l^{-1}, equaling 7.35 mmol phosphate l^{-1}, was found to ensure a non-limiting P supply of *A. adeninivorans* in SYN6-MES under the culture conditions applied in this study.

Systematic approach to characterize growth as a function of pH

In order to provide a solid basis for the selection of appropriate pH conditions for *A. adeninivorans* cultures, the growth of *A. adeninivorans* as a function of pH was characterized. For this purpose, *A. adeninivorans* was cultured at a broad range of pHs in differently buffered SYN6 derivatives (see Table 1). Monitored OTR values served for the mathematical derivation of pH and specific growth rate over fermentation time which were plotted as specific growth rate over pH. The approach to the determination of pH over fermentation time is explained in the following [47].

The growth-coupled oxygen consumption over fermentation time $c_{O_2}(t)$ served as guiding variable, and is obtained by the integration of the OTR (Eq. 1).

$$c_{O_2}(t) = \int_0^t \mathrm{OTR}\,\mathrm{d}t \tag{1}$$

The acidification of culture media containing ammonium as N source, such as the SYN6 derivatives, is caused by the growth-coupled ammonium consumption over fermentation time $c_{NH_4^+}(t)$ [4]. Biomass formation, oxygen consumption, and ammonium consumption are assumed to be linearly correlated during growth. Thus, an ammonium-dependent biomass yield Y_{X/NH_4^+} can be calculated according to the biomass composition of yeast (Eq. 2, [3]). This yield was confirmed by investigations on N utilization of *A. adeninivorans* (data not shown). Additionally, the total biomass formation CDW_{total} can be correlated to the total oxygen consumption $c_{O_2,total}$ of the cultures, yielding the oxygen-dependent biomass yield Y_{X/O_2} (Eq. 3).

$$Y_{X/NH_4^+} = 158.7\,\mathrm{g\ CDW\ mol\ N^{-1}}, \tag{2}$$

$$Y_{X/O_2} = \frac{\mathrm{CDW_{total}}}{c_{O_2,\,\mathrm{total}}}. \tag{3}$$

The combination of Eqs. (1) to (3) results in the ammonium consumption over fermentation time $c_{NH_4^+}(t)$ (Eq. 4), which now can be calculated from the time-dependent oxygen consumption $c_{O_2}(t)$ as defined by Eq. (1)

$$c_{NH_4^+}(t) = \frac{Y_{X/O_2}}{Y_{X/NH_4^+}} \cdot c_{O_2}(t). \tag{4}$$

The growth-coupled assimilation of ammonium leads to the equimolar net release of protons H^+ into the medium (Eq. 5, [27, 40, 47]). The resulting acidification is buffered by the employed buffer compounds of the SYN6 derivatives according to Eq. (6), with *HA* and A^- being the molecular buffer species

$$c_{NH_4^+}(t) = c_{H^+}(t), \tag{5}$$

$$A^- + H^+ \rightleftarrows HA. \tag{6}$$

The buffering of pH is assumed to follow the equation of Henderson and Hasselbalch. According to this equation, pH over fermentation time pH(t) can be calculated as the function of the dissociation constant pK_a and the concentrations of the buffer species over fermentation time $c_{HA}(t)$ and $c_{A^-}(t)$ (Eq. 7)

$$\mathrm{pH}(t) = \mathrm{p}K_a - \log\frac{c_{HA}(t)}{c_{A^-}(t)}. \tag{7}$$

The quantitative conversion of the buffer species can be determined by Eqs. (8), (9)

$$c_{HA}(t) = c_{HA}(0) + c_{H^+}(t), \tag{8}$$

$$c_{A^-}(t) = c_{A^-}(0) - c_{H^+}(t). \tag{9}$$

Thus, pH over fermentation time pH(t) can be described as the function of the ammonium consumption over fermentation time $c_{NH_4^+}(t)$ by inserting Eqs. (5), (8), and (9) into Eq. (7) (Eq. 10) [47].

$$\mathrm{pH}(t) = \mathrm{p}K_a - \log\frac{c_{HA}(0) + c_{NH_4^+}(t)}{c_{A^-}(0) - c_{NH_4^+}(t)} \tag{10}$$

The initial concentrations of the buffer species $c_{HA}(0)$ and $c_{A^-}(0)$ in Eq. (10) are unknown. According to Eq. (7) they can be described as Eq. (11). Corresponding to the total buffer concentration of the media c_{HA+A^-}, they are also defined as Eq. (12).

$$\frac{c_{HA}(0)}{c_{A^-}(0)} = 10^{\mathrm{p}K_a - \mathrm{pH}(0)}, \tag{11}$$

$$c_{HA+A^-} = c_{HA}(0) + c_{A^-}(0) \tag{12}$$

Thus, the initial concentrations of the buffer species can be calculated by combining Eqs. (11) and (12), which results in Eqs. (13) and (14)

$$c_{\mathrm{HA}}(0) = \frac{10^{\mathrm{p}K_{\mathrm{a}}-\mathrm{pH}(0)}}{10^{\mathrm{p}K_{\mathrm{a}}-\mathrm{pH}(0)}+1} \cdot c_{\mathrm{HA+A^-}}, \tag{13}$$

$$c_{\mathrm{A^-}}(0) = \frac{1}{10^{\mathrm{p}K_{\mathrm{a}}-\mathrm{pH}(0)}+1} \cdot c_{\mathrm{HA+A^-}}. \tag{14}$$

Now pH over fermentation time pH(t) can be calculated by inserting Eqs. (13) and (14) into Eq. (10) as the function of the initial pH of the SYN6 derivatives pH(0), the dissociation constant of the employed buffer pK_{a}, the total buffer concentration $c_{\mathrm{HA+A^-}}$, and the time-dependent course of ammonium consumption $c_{\mathrm{NH_4^+}}(t)$ according to Eq. (4).

The cultivation series for the determination of the specific growth rate as a function of pH was comprised of 17 shake-flask cultures in SYN6 derivatives under different pH conditions, alternatively buffered as denoted in Table 1. The cultivations were conducted under non-limiting standard conditions as described in the section "Materials and methods". Initial pHs pH(0) and buffer concentrations $c_{\mathrm{HA+A^-}}$ were adjusted ensuring a pH decrease of less than 2 U during cultivation. Each buffer covered a distinct range of culture pHs corresponding to its dissociation constant pK_{a} within a total range of culture pHs between 2 and 8. Predictive modeling of pH over fermentation time pH(t) according to the above considerations (Eqs. 1–14) served as tool for the adjustment of the buffering [pH(0), pK_{a}, $c_{\mathrm{HA+A^-}}$].

Figure 4 depicts examples of the cultivations conducted under relatively acidic (a) and alkaline (c) conditions, and an example conducted under intermediate pH conditions (b). The cultures exhibited RQs between 1.1 and 1.2 during growth (data not shown), indicating a stable aerobic metabolism. They exhibited consistent biomass yields and coherent material balances. Hence, favorable conditions for growth only depending on the adjusted pH conditions were provided. Measured and calculated pHs diverged 0.1 U during growth at most (Fig. 4a–c). Thus, the values were of a close match, validating the applied method of pH calculation (Eqs. 1–14). The specific growth rates of the cultures were derived from the OTRs based on growth-coupled oxygen consumption. The specific growth rate of the phosphate-buffered culture increasingly declined from 0.32 $\mathrm{h^{-1}}$ to lower values due to increasingly growth-inhibiting pHs lower than 2.8 (hour 11) (Fig. 4a). In contrast, a pH range of 5.2–4.1 provided non-inhibiting conditions, since the HOMOPIPES-buffered culture depicted by Fig. 4b exhibited a specific growth rate of about 0.32 $\mathrm{h^{-1}}$ throughout growth. The specific growth rate of the HEPES-buffered culture shown in Fig. 4c increased from 0.18 to 0.27 $\mathrm{h^{-1}}$

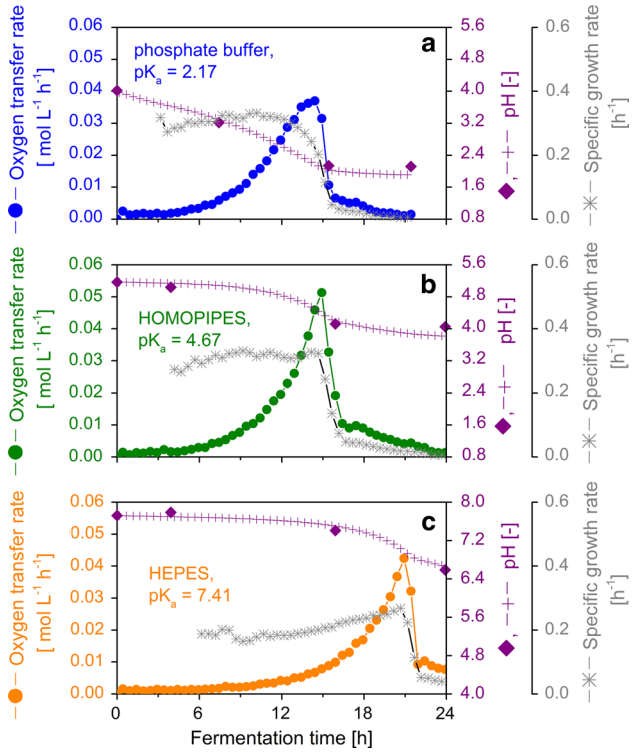

Fig. 4 *A. adeninivorans* LS3 cultures in differently buffered SYN6 derivatives. Employed buffers: Phosphate (pK_{a} = 2.17, 120 mmol $\mathrm{l^{-1}}$, initial pH 4.0) (**a**), HOMOPIPES, identical to the culture shown in Figs. 1b, d (pK_{a} = 4.67, 140 mmol $\mathrm{l^{-1}}$, initial pH 5.2) (**b**), and HEPES (pK_{a} = 7.41, 140 mmol $\mathrm{l^{-1}}$, initial pH 7.7) (**c**). pH (*pink crosses*) and specific growth rate (*grey stars*) over fermentation time were derived from the respective OTR courses of the cultures in **a**–**c**. Initial, error-prone specific growth rates were omitted due to the underlying high relative errors at low OTRs. Measured pHs (*pink diamonds*) serve for the validation of the calculated pH courses. Operating conditions (for a non-limiting oxygen supply): orbital shaking diameter 50 mm, shaking frequency 350 rpm, shake flask 250 ml, liquid volume 10 ml, temperature 30 °C

until the stationary phase was reached. This culture grew itself from an initially suboptimal alkaline pH of 7.7 to a better suited (in this case: lower) pH of 6.8.

The extensive data on pH and specific growth rate over fermentation time of the cultivation series (Table 1) were plotted as specific growth rate over pH for the individual cultures, each plot representing a distinct part of a pH optimum curve for growth. Figure 5 merges all data to result in the function of specific growth rate (μ) over the full range of growth-relevant acidic to alkaline pH values.

The set of data points was fitted by a function (Eq. 15) according to a model of Qin and Cabral [39]. The respective bell-shaped fitting curve (Fig. 5) was obtained by the estimation of the maximal specific growth rate at optimal pH $\mu_{\max}$, the inhibition constants $K_{I1,2}$, and the empirical variables $z_{1,2}$ (relative least square method).

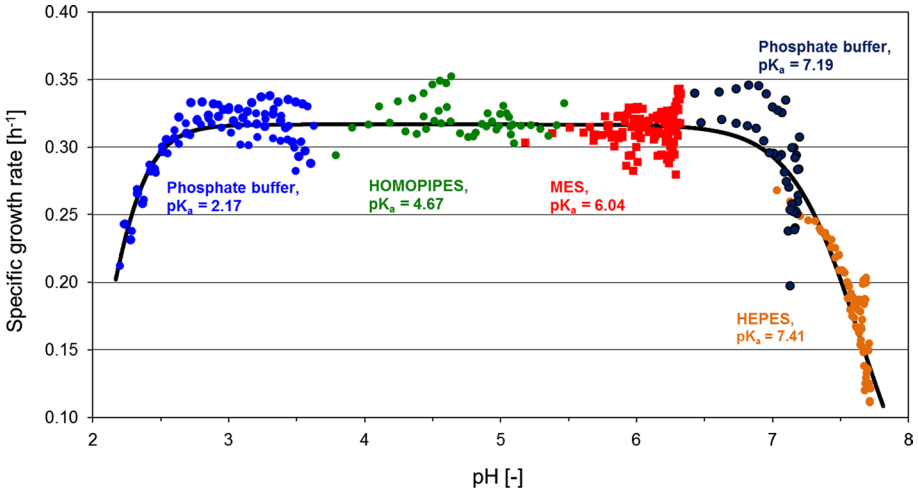

Fig. 5 Specific growth rate as a function of pH for *A. adeninivorans* LS3. The time-dependent courses of pH and specific growth rate of shake-flask cultures were plotted as specific growth rate over pH. The series comprised 17 cultures in SYN6 derivatives under different pH conditions either buffered with phosphate ($pK_a = 2.17$), HOMOPIPES ($pK_a = 4.67$), MES ($pK_a = 6.04$), phosphate ($pK_a = 7.19$), or HEPES ($pK_a = 7.41$) (see diagram, Table 1, respectively). Figure 4a–c denote three exemplary cultures of this series. The resulting set of specific growth rate over pH data (see diagram) was fitted by a function according to Qin and Cabral [39] (Eq. 15, *grey line*). The underlying parameters were estimated as: $z_1 = 2.75$, $z_2 = 1.69$, $K_{I1} = 0.0083$ mol l^{-1}, $K_{I2} = 2.25 \times 10^{-8}$ mol l^{-1}, and $\mu_{max} = 0.32\ h^{-1}$

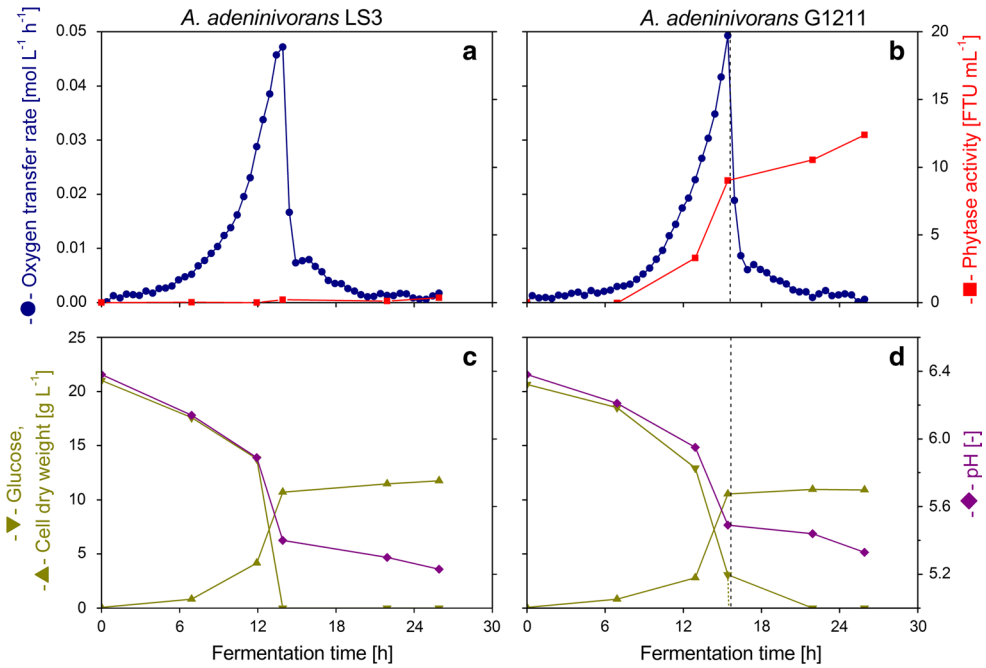

Fig. 6 *A. adeninivorans* LS3 (**a**, **c**) and *A. adeninivorans* G1211 (**b**, **d**) cultures in SYN6-MES. Courses of OTR and phytase activity (**a**, **b**). Courses of glucose, cell dry weight, and pH (**c**, **d**). *Vertical dashed line* in **b**: estimated time of glucose depletion, indicated by the sharp drop of the OTR, *dotted line* in **d**: estimated glucose concentration before depletion. Operating conditions (for a non-limiting oxygen supply): orbital shaking diameter 50 mm, shaking frequency 350 rpm, shake flask 250 ml, liquid volumes 10 ml, temperature 30 °C

$$\mu = \mu_{max} \cdot \frac{1}{1 + \left(\frac{10^{-pH}}{K_{I1}}\right)^{z_1} + \left(\frac{K_{I2}}{10^{-pH}}\right)^{z_2}}. \quad (15)$$

The average deviation of the data points from the fitted curve is 0.01 h^{-1}. The maximal specific growth rate μ_{max} is fitted as 0.32 h^{-1} and, thus, is of close match to the individually obtained maximal specific growth rates of the cultures (Figs. 1a–d, 2, 3, 4a, b). The pH optimum at the maximal specific growth rate is characterized by a plateau between acidic pHs of 2.8 and 6.5 and by a rapid decline of the specific growth rate at pHs outside of this range. Similar pH optimum curves were observed for *H. polymorpha* and other industrially employed fungi [9, 46].

Cultivation of *A. adeninivorans* G1211 in Syn6-MES

Arxula adeninivorans G1211 was cultivated in Syn6-MES to prove whether the medium composition that is optimal for the growth of the wild-type strain *A. adeninivorans* LS3 is also suited for the cultivation of this genetically modified strain. Figure 6a, b show the OTR-curves and phytase activity of the strains LS3 and G1211, respectively.

Both strains show an oxygen non-limited growth and reach a maximal OTR of about 0.05 mol l^{-1} h^{-1}. In contrast to the similar OTR of both cultivations, the phytase activity differs strongly. Whereas the phytase activity of the cultivation of G1211 reaches about 12 FTU ml^{-1} after 24 h, hardly any phytase activity can be observed during the cultivation of strain LS3. Figure 6c, d show pH, glucose and cell dry weight concentration of the cultivations of *A. adeninivorans* LS3 and G1211, respectively. In both cultivations, glucose is exhausted after about 15 h and a cell dry weight of about 12 g l^{-1} is reached. The courses of the pH values are also similar; they decrease from an initial value of 6.4–6.0 after about 12 h and drop to ca. 5.5 after 14 h. Until the end of the cultivations, both pH values decrease to ca. 5.25. This shows that the applied cultivation conditions and the Syn6-MES medium are also suited for the non-limited growth of an *A. adeninivorans* strain producing a heterologous protein.

Conclusions

The rational definition of conditions for a non-limiting oxygen and phosphorus supply as well as the pH stabilization of the medium to non-inhibiting values provide basic conditions for *A. adeninivorans* cultures characterized by short fermentation times, a complete aerobic metabolism without anaerobic byproducts or overflow-metabolites, maximized growth rates, and maximized biomass yields. Thereby, the basis for strain screening is laid. Different strains can be cultivated in this optimized medium at appropriate conditions and the strain most suitable for the respective application can be selected. Additionally, pH-stabilized SYN6-MES medium, derived from SYN6 medium to be employed in shaken cultures, provides favorable conditions for a screening, supporting process consistency with future technical-scale fermentations in SYN6 medium. The identification of general growth characteristics (e.g., growth rates, biomass yields, phosphorus demand) provides valuable references for the layout of processes. In this respect, the pH optimum curve deduced in this study can serve as basis for the selection of appropriate pH conditions for *A. adeninivorans* cultures. The mathematical descriptions of pH over fermentation time and specific growth rate over pH represent useful modules for the predictive modeling of pH-dependent growth of *A. adeninivorans*. The modelings are found to reproduce experimental data well. The presented approach, employing simple assumptions and a limited number of cultivations at easy-to-handle shake flask scale, demonstrate an effective method for the analysis of growth characteristics and the rational definition of culture conditions. The results with the wild-type strain LS3 were proven by studying heterologous gene expression (phytase) in a recombinant *A. adeninivorans* strain under defined culture conditions.

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