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Effect of oxygen supply on passaging, stabilising and screening of recombinant *Hansenula polymorpha* production strains in test tube cultures

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Abstract

Twenty-four *Hansenula polymorpha* transformants were passaged and stabilised in glucose medium and screened in glycerol medium for recombinant phytase in shaken test tubes. The cultivations were performed under either limited or non-limited oxygen supply. Maximum oxygen transfer capacities of test tubes were assessed by sulfite oxidation. Oxygen-limited glucose cultures resulted in a partially anaerobic metabolism and formation of 4.1 g ethanol l^{-1}, which was subsequently aerobically metabolised. Non-limited oxygen supply led to overflow metabolism and to accumulation of 2.1 g acetic acid l^{-1}, reducing the biomass yield. The use of glycerol in the screening main cultures prevented by-product formation irrespective of oxygen supply. Preculturing in glucose medium under non-limited oxygen supply resulted in a 20-h lag phase of the screening main culture. This lag phase was not observed when preculturing was performed under oxygen limitation. Phytase activity was on average 25% higher in cultures passaged, stabilised and screened under limited oxygen supply than in cultures under non-limited oxygen supply.

Keywords: *Hansenula polymorpha*; Oxygen transfer rate; Phytase; Screening; Sulfite oxidation; Test tube culture

1. Introduction

Over the last decades biotechnologists have developed powerful methods for the efficient production of recombinant proteins in a range of micro-organisms, including prokaryotes such as *Escherichia coli* [1,2] and several eukaryotic micro-organisms. The latter provide post-translational modifications according to a general eukaryotic pattern, which is often essential for compounds considered for administration to humans. Heterologous gene expression has been assessed in yeasts such as *Saccharomyces cerevisiae* or methylotrophic species such as *Pichia pastoris* and *Hansenula polymorpha* [3–9]. Product examples include *S. cerevisiae*-derived insulin [2] and hepatitis B surface antigen vaccines, produced in *H. polymorpha* and *S. cerevisiae* [10]. The economic significance of such recombinant therapeutics enforces efficient industrial production processes, which depend to a large extent on the performance of the respective production strains and the design of efficient upstream and downstream procedures.

Shaken bioreactors are widely used for strain development. They are inexpensive and the reactors can be operated in parallel in large numbers [11]. In industrial biotechnology up to several hundred thousand individual experiments per company are run every year [12]. Nevertheless, the design of culture conditions is frequently based on insufficient empirical results and phenomenological observations. One reason is a lack of appropriate measuring methods for shaken bioreactors [11]. The careful adjustment of oxygen supply during passaging, stabilisation and screening of strains is often neglected, although most products of higher value are obtained from aerobic micro-organisms [11] with oxygen as a universal substrate

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and a multiple effector [13,14]. The influence of oxygen supply on metabolism and product formation has frequently been reported [15–18]. For instance, Saito et al. [19] have reported significantly higher biotin production in recombinant *Sphingomonas* sp. pSP304 in test tube cultures than in an oxygen-controlled jar fermenter. By applying special agitation conditions biotin production could be approximated to that in the test tube cultures. Biotin production correlated with the k_La value. This case study demonstrated the impact of defined oxygen supply conditions in shaken cultures for preventing selection of inferior strains and inconsistent productivities between screening and the production process.

The generation of recombinant *H. polymorpha* strains is achieved by transformation with heterologous expression vectors [9,20], resulting in a variety of hundreds of recombinant clones, initially carrying the vectors as episomes [21,22]. Transformants are selected by complementing an auxotrophy of the uracil-auxotrophic host strain RB11 [22] by the incorporated *URA3* gene present on the plasmid. Subsequently, several passaging cultivations are carried out under selective conditions, representing a sequence of 40–60 generations. Heterologous gene expression is prevented by using glucose, a carbon source repressive for the promoter that drives the expression. Repression is essential, because heterologous gene expression may cause a negative selection pressure during strain generation. During passaging the expression vector integrates in varying copy numbers into the genome of host strains [20,23]. The newly acquired expression characteristics depend on various parameters, such as the integration locus (position effect) [24,25] or the copy number of the integrated foreign DNA (gene dosage effect) [21,22,24,25].

Vectors of episomal location potentially still present after passaging are lost during stabilisation in cultures in non-selective medium [23]. Non-recombinant cells arising during stabilisation grow on without being selected. These cells are eliminated in subsequent cultures under selective conditions. The remaining transformants are mitotically stable with the expression vectors integrated into the genome.

In the present study *H. polymorpha* transformants were screened for production of recombinant phytase under control of the strong formate dehydrogenase (*FMD*) promoter. The promoter was derepressed in glycerol cultures at ≤ 4 g glycerol l^{-1} [6,23]. Glucose preculturing repressed the promoter and thus prevented an early phytase formation. Phytase is a feed additive and releases phosphate from phytic acid, enhancing the availability of phosphorus.

We investigated the influence of oxygen supply on the development of recombinant *H. polymorpha* strains. For this purpose passaging, stabilisation and screening were carried out in test tube cultures under defined conditions of limited and non-limited oxygen supply. The cultures were analysed by a parallel on-line measurement technique for the oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) in shaken flasks [26].

2. Materials and methods

2.1. Measurement of relative maximum liquid height

A test tube was fixed within a tiltable holder on a shaking machine with an orbital diameter of 50 mm (LabShaker, Kühner, Basel, Switzerland). The rotating liquid meniscus inside the test tube filled with an ink/water solution was observed by a CCD camera (DCR-TRV890E, Sony, Germany), which was also mounted on the shaking machine. The liquid height of the meniscus was measured by a scale placed in parallel to the test tube axis. The relative maximum liquid height (MLH) was defined as the distance between the non-agitated liquid surface and the top of the agitated liquid meniscus. The average deviation of the MLH for different filling volumes at constant shaking frequency and tilting angle was defined as the mean of the absolute deviations from the average MLH of the different filling volumes.

2.2. Measurement of the OTR_{max} by sulfite oxidation

OTR_{max} is defined as the maximum gas–liquid oxygen transfer capacity per liquid volume V_L and time (mol l^{-1} h^{-1}) and was determined by the operating conditions and the physicochemical properties of the liquid system [27]. For the OTR_{max} measurements, test tubes were filled with defined liquid volumes of a solution with 0.5 mol sulfite l^{-1} as reported [28]. Depletion of sulfite is accompanied by a drop of the pH value, which can be followed by the colour change of a pH indicator. Colour change was recorded by a camera (DCR-VX700E, Sony, Germany). OTR_{max} is proportional to the length of the sulfite oxidation reaction time until sulfite depletion. Oxygen transfer velocity is defined as $OTR_{max} \cdot V_L$ (mmol h^{-1}), which is equal to the molar flow rate of oxygen transferred into liquid. The average deviation of the oxygen transfer velocities for different filling volumes at constant shaking frequency and tilting angle was defined as described for the relative maximum liquid height (Section 2.1).

2.3. Correlation factor K between sulfite solution and YNB medium

Factor K was used for converting the OTR_{max} of the sulfite system to the OTR_{max} of the YNB medium. OTR_{max} is proportional to the specific mass transfer area a, and the diffusion coefficient D_{O2} and the oxygen solubility L_{O2}. In unbaffled shaken bioreactors the specific mass transfer area a is only a function of operating conditions [14], and D_{O2} and L_{O2} depend on the ionic strength of the liquid system [29,30]. Thus, according to Eq. 1 a

fixed correlation factor K between the OTR_{max} of the sulfite solution and the YNB medium under equal operating conditions (a = constant) can be estimated. Maier et al. [31] have shown that a constant correlation factor can be used for a broad range of operating conditions.

$$K = \frac{OTR_{max}\ (YNB)}{OTR_{max}\ (sulfite)} = \frac{aD_{O_2}(YNB)\ L_{O_2}(YNB)}{aD_{O_2}(sulfite)\ L_{O_2}(sulfite)} = \text{constant} \quad (1)$$

The OTR_{max} of the YNB medium was obtained from the OTR plateau of the oxygen-limited culture of Fig. 3A, filled squares [27]. The OTR_{max} of the sulfite solution at the same operating conditions was measured as described above [31].

2.4. On-line measurement of the OTR and CTR

The device for the on-line measurement of the OTR, CTR and RQ in shaken flasks is described in [26]. The cultivations were conducted in specially designed measuring flasks, ensuring hydrodynamics and gas phase conditions identical to those in normal cultures in Erlenmeyer flasks with cotton plugs. This device has already successfully been used in several previous projects [28,32,33].

2.5. Expression vector

The *H. polymorpha* expression vector pFPMT121 was used for transformation [34]. A reporter gene similar to that described by Mayer et al. [6] encoding a phytase mutein including a signal sequence was inserted into the *Eco*RI site of the expression cassette. Constructs contained the *S. cerevisiae*-derived orotidine-5′-phosphate decarboxylase (*URA3*) gene for selection. Plasmid constructs were propagated in *E. coli* strain HB101 [35]. *E. coli* was grown in LB medium, which contained per litre 10 g Bacto-tryptone (Becton Dickinson, Franklin Lakes, MD, USA), 10 g Bacto-yeast extract (Becton Dickinson) and 10 g NaCl. LB medium was supplemented with 100 mg ampicillin when required for selection. Plasmid DNA was prepared by the alkaline extraction procedure [36].

2.6. Transformation

H. polymorpha RB11 (*ura3*) served as host and was grown in YPD. YPD contained per litre 10 g yeast extract (Roth, Karlsruhe, Germany), 10 g peptone 190 (Gibco, Invitrogen, Karlsruhe, Germany) and 20 g glucose. *H. polymorpha* RB 11 was transformed as reported in [22]. Transformants were selected for uracil prototrophy on YNB-glucose plate cultures. YNB-glucose plates contained per litre 18 g agar, 20 g glucose, 1.4 g yeast nitrogen base without ammonium and amino acids (Becton Dickinson) and 13.3 g $NH_4H_2PO_4$.

2.7. General culture conditions

The passaging, stabilising and screening cultures were shaken on an orbital shaking machine with 50 mm shaking diameter (LabShaker, Kühner). The cultivations were carried out at 37°C in test tubes with an inner diameter of 14 mm and a length of 100 mm (Schütt Labortechnik, Göttingen, Germany). Aluminium caps (Labocap 17/18, Schütt Labortechnik) served as sterile closure. The test tubes were filled with 3 ml of medium. Each transformant was passaged, stabilised and screened independently under conditions of non-limited or limited oxygen supply. For this purpose the OTR_{max} of the test tube cultures was adjusted as described in Section 3.1. In parallel to the test tube cultures, culturing was performed in unbaffled 250-ml measuring flasks [26]. Additionally, standard Erlenmeyer flask cultures were handled in parallel and harvested for off-line analysis of culture parameters. Flask cultures were conducted at OTR_{max} ensuring an oxygen supply identical to that in the test tube cultures. All cultures were inoculated, if not otherwise specified, with broth of a predecessor culture, using one hundred-fiftieth of the final culture volume.

2.8. Passaging

Twenty-four colonies of transformants were randomly selected from YNB-glucose plate cultures and cultivated in test tubes with YNB-glucose medium. After 48 h each of the grown cultures was used for inoculation of test tubes with fresh YNB-glucose medium. This cultivation was repeated in eight successive steps as shown in Fig. 1A. YNB-glucose medium was prepared according to Section 2.6 without adding agar. Initial pH of YNB-glucose medium was adjusted to 4.1 with 1 M NaOH.

2.9. Stabilisation

After the first, third, fifth and eighth passages each transformant was cultivated in two successive test tube cultures with YPD medium for 48 h as shown in Fig. 1B. Non-recombinant cells were subsequently selected out in YNB-glucose cultures for 48 h. Stabilised strains were stored on YPD plate cultures. YPD was prepared according to Section 2.6. For plates YPD was supplemented with 18 g agar l^{-1}.

2.10. Screening

Stabilised transformants of the first, third, fifth and eighth passages were screened for phytase production as depicted in Fig. 1C. Transformants were precultured in YNB-glucose medium. Screening was carried out in YNB-glycerol cultures for 48 h. YNB-glycerol medium contained per litre 15 g glycerol, 1.4 g yeast nitrogen base without ammonium and amino acids (Becton Dick-

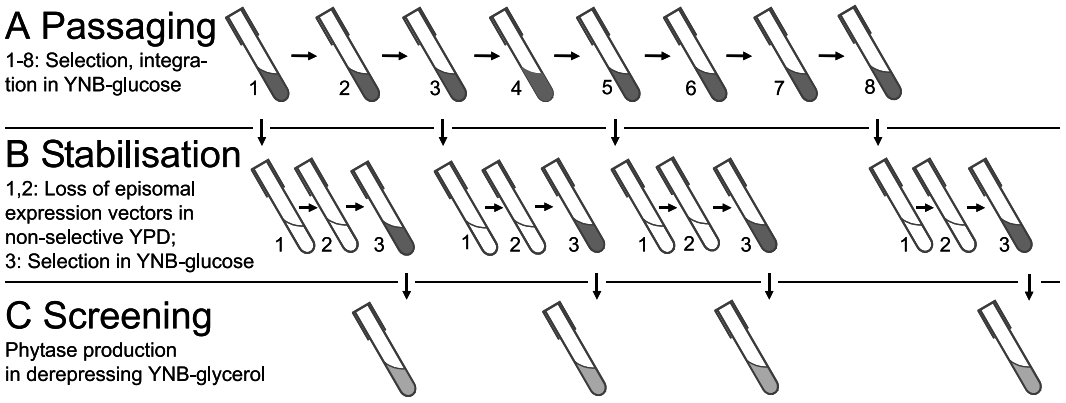

Fig. 1. Passaging, stabilisation and screening in test tube cultures for each of 24 transformants. Cultivations were carried out under conditions of limited oxygen supply ($OTR_{max} = 0.012$ mol l^{-1} h^{-1}) as well as non-limited oxygen supply ($OTR_{max} = 0.081$ mol l^{-1} h^{-1}). Transformants were passaged in eight successive selective YNB-glucose cultures (A). After passages 1, 3, 5 and 8 transformants were stabilised in two successive non-selective YPD cultures. Finally, cells without expression vector were selected in a further YNB-glucose culture (B). Stabilised transformants were screened for phytase production after cultivation in YNB-glycerol medium (C).

inson) and 13.3 g $NH_4H_2PO_4$. Initial pH was adjusted to 4.1 with 1 M NaOH. Phytase activity was measured after cultivation.

2.11. Culture analytics

The biomass was determined as cell dry weight (CDW) by filtering 8 ml culture broth through dried and preweighed cellulose acetate filters, pore size 0.2 μm (11107-47-N, Sartorius, Göttingen, Germany). The filter residue was resuspended once in 9 g NaCl l^{-1}, filtrated again and dried on the filter at 105°C until the mass remained constant. Glucose, ethanol and acetic acid were measured by high-performance liquid chromatography (HPLC) (Dionex Softron, Germering, Germany). Of the filtrated culture samples (0.22 μm, CM, Qualilab, Merck, Darmstadt, Germany), 20 μl was eluted with 1 mM sulfuric acid (flow rate 0.6 ml min^{-1}). An organic acid resin 250×8 mm (Art. No. 528980, CS Chromatographie Service, Langerwehe, Germany) served as stationary phase. Compounds were detected by a UV detector (UVD340S, Dionex Softron) and a refractometer (RI-71, Dionex Softron).

Phytase activity was measured kinetically, based on the hydrolysis of *p*-nitrophenylphosphate to phosphate and *p*-nitrophenol. To initiate the reaction, 100 μl of 5 mM *p*-nitrophenylphosphate was added to a mixture of 50 μl of 0.8 M acetic acid (pH 5.0) and 50 μl of the filtrated culture sample (0.22 μm, CM, Qualilab, Merck) or 50 μl of a phytase standard. The rate of *p*-nitrophenol formation was determined by the linear change in absorbance at 410 nm (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany). Phytase activity was expressed as phytase units (FTU) and was defined as enzyme amount releasing 1 μmol of inorganic phosphate per min from sodium phytate at pH 5.5 at 37°C. For standardisation phytase with 5450 FTU g^{-1} (Lohmann Animal Health, Cuxhaven, Germany) was used.

3. Results and discussion

3.1. Investigation of hydrodynamics and OTR_{max} in test tubes

Hydrodynamics and mass transfer in shaken Erlenmeyer flasks are fairly well characterised [12,27,37,38]. Less is known about hydrodynamics and mass transfer in shaken test tubes, although these are frequently used as small-scale cultivation vessels [19,39–42]. Therefore hydrodynamics and mass transfer were characterised prior to the investigations with the *H. polymorpha* test tube cultures.

Fig. 2A depicts the MLH of the liquid meniscus for varying shaking frequencies and tilting angles (0°, 10°, 20°, 30°). MLHs were averaged for the filling volumes of 2, 3 and 4 ml. The average deviations are shown for each shaking frequency and tilting angle.

In vertically shaken test tubes the liquid meniscus rotates as a symmetric paraboloid, similar to those observed in a shaken flask [27]. Thus, similar hydrodynamic regimens for vertical test tubes and shaken flasks can be suggested. Vertical test tubes (0°) led to an exponential increase of MLH with increasing shaking frequency (Fig. 2A).

Compared to shaken flasks, test tubes can easily be tilted and thus provide an additional operating parameter. Tilting caused an asymmetrically rotating liquid meniscus. Tilted glass walls force the liquid meniscus out of symmetric rotation, presumably having similar effects as baffles in shaken flasks [14,43]. Tilting caused sigmoidal MLH profiles in correlation to shaking frequency (Fig. 2A). The reason for this is still under investigation. The initial exponential slope of MLH increased with increasing tilting angle (Fig. 2A). Thus, tilting enlarged the extension of the liquid meniscus compared to vertically shaken test tubes. MLH was found to be largely independent of filling volume (Fig. 2A). Thus, the extension of the liquid meniscus

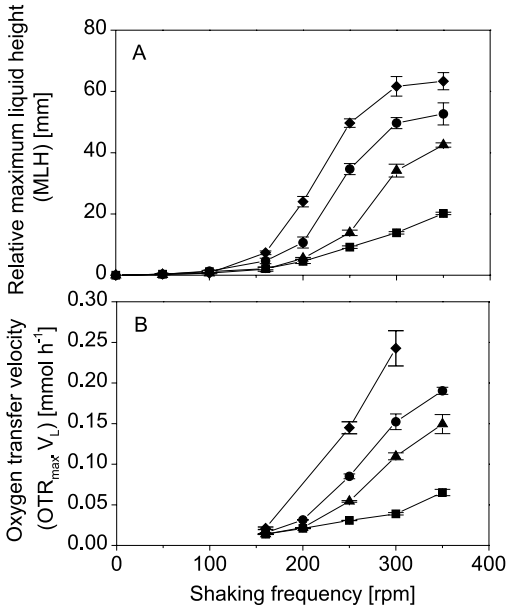

Fig. 2. MLH of the rotating liquid meniscus in test tubes (A) and OTR_{max}·V_L (B) as functions of shaking frequency for tilting angles of 0° (■), 10° (▲), 20° (●) and 30° (◆) and for a shaking diameter of 50 mm. Values are averaged for 2, 3 and 4 ml filling volume, bars = average deviations. MLH of the rotating liquid meniscus of an ink/water solution was measured by means of a CCD camera (DCR-TRV890E, Sony). OTR_{max}·V_L was measured by sulfite oxidation according to a method described in [28].

was obviously exclusively dependent on tilting angle and shaking frequency, which determine the forces acting on the liquid meniscus. The bulk part of the liquid, filling the cylindrical shaft of the test tube, was obviously not involved in the formation of the meniscus shape. Thus, increase of filling volumes solely shifted the position of the liquid meniscus along the tube axis according to the displacement by the liquid volume. At higher tilting angles and shaking frequencies the average deviations of MLH for different filling volumes increased (Fig. 2A). This was probably due to the fact that the rotating meniscus increasingly reached the bottom of the test tube, especially at lower filling volumes.

Fig. 2B depicts the OTR_{max}·V_L for varying shaking frequencies and tilting angles (0°, 10°, 20°, 30°). OTR_{max}·V_L were averaged for the filling volumes of 2, 3 and 4 ml. The average deviations are shown for each shaking frequency and tilting angle. OTR_{max}·V_L increased, just like the MLH, with increasing shaking frequency and tilting angle (Fig. 2B). OTR_{max}·V_L was also widely independent of filling volume, as shown by the average deviations (Fig. 2B). OTR_{max}·V_L and MLH were linearly correlated within the MLH range of 0–60 mm (data not shown). This is probably due to the predominant effect of the specific mass transfer area a of the liquid meniscus on gas/liquid mass transfer. The mass transfer area increased with MLH and thus proportionally raised OTR_{max}. A very similar trend had already been shown for microtitre plates by Hermann et al. [44]. Thus, suitable OTR_{max}·V_L can be adjusted guided by the MLH, no matter whether obtained by a low shaking frequency and a high tilting angle or vice versa. Additionally, OTR_{max} can easily be extrapolated from one filling volume to the other, because OTR_{max}·V_L is widely constant at equal shaking frequencies and tilting angles. High OTR_{max} as known for shaken flasks can be achieved in test tubes. A filling volume of 2 ml at 300 rpm yielded an OTR_{max} of 0.14 mol l^{-1} h^{-1} (Fig. 2B).

From the data pool of Fig. 2B operating conditions were selected providing OTR_{max} for a limited or non-limited oxygen supply in the test tube cultures during passaging and screening in YNB medium. The OTR_{max} of the sulfite solutions was converted to the OTR_{max} of YNB medium by means of the correlation factor K (Section 2.3), resulting in $K = 1.5$.

To enable an on-line monitoring of the OTR and CTR in the test tube cultures, it was necessary to adjust operating conditions for 250-ml shaken flasks to identical characteristics. Therefore the OTR_{max} of the test tubes was transferred to shaken flasks by a rational choice of operating conditions calculated by equations given in [31]. Table 1 depicts the respective operating conditions for test tubes and shaken flasks. OTR_{max} for limited oxygen supply was approximated precisely to ensure the same levels of limitation in test tube and shaken-flask cultures. Non-limited oxygen supply is ensured as long as OTR_{max} is distinctly higher than the maximal respiration rate of the culture [27]. Thus, OTR_{max} for non-limited oxygen supply in shaken flasks was allowed to be lower than in test tubes as shown in Table 1.

Table 1
Operating conditions ensuring limited and non-limited oxygen supply in shaken test tubes and 250-ml shaken flasks

	Test tube		250-ml shaken flask	
	Limited oxygen supply	Non-limited oxygen supply	Limited oxygen supply	Non-limited oxygen supply
Tilting angle (°)	30	20	0	0
Shaking frequency (rpm)	160	320	240	240
Filling volume (ml)	3	3	55	10
OTR_{max} (sulfite) (mol l^{-1} h^{-1})	0.008	0.052	0.008	0.028
OTR_{max} (YNB) (mol l^{-1} h^{-1})	0.012	0.081	0.012	0.042

Operating conditions and OTR_{max} for shaken flasks were calculated as reported by Maier et al. [31]. Operating conditions and OTR_{max} for test tubes were extracted from OTR_{max} measurements based on sulfite oxidation as shown in Fig. 2B. OTR_{max} of sulfite solution was converted to OTR_{max} of YNB medium by a constant correlation factor $K = 1.5$ as defined by Eq. 1.

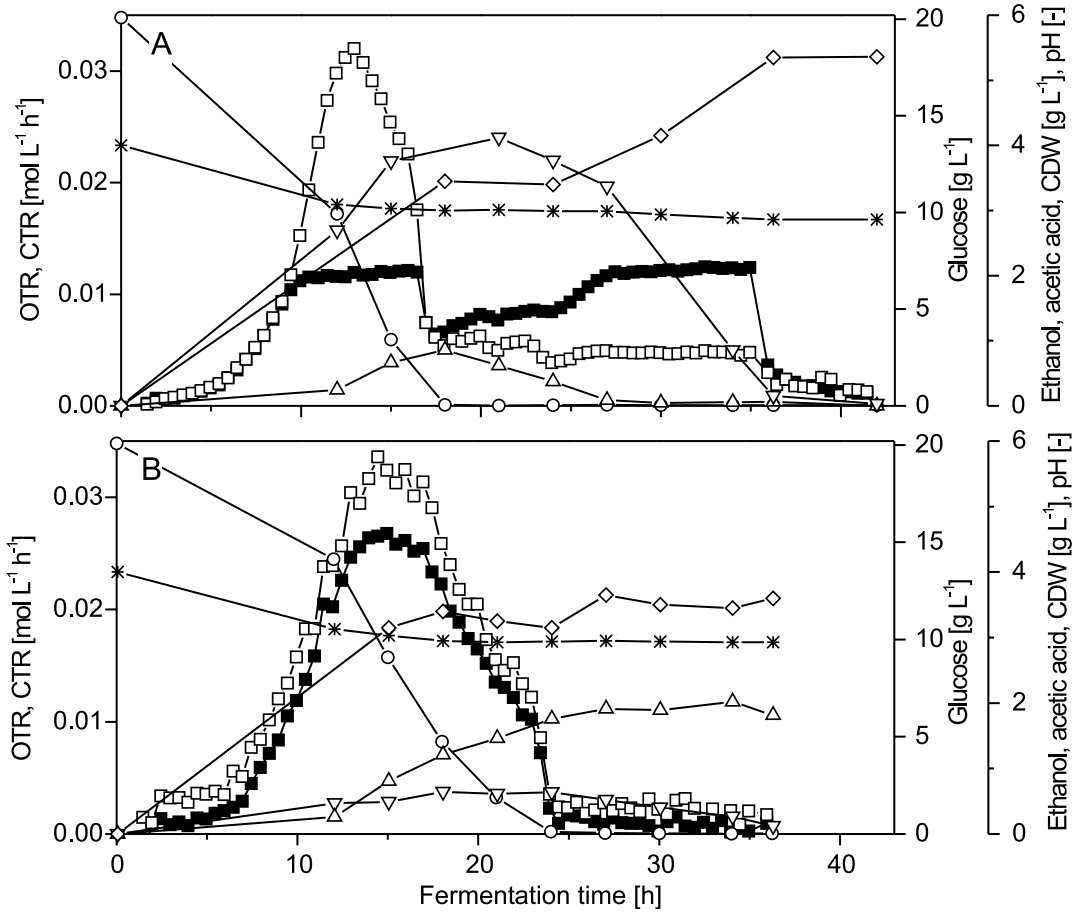

Fig. 3. Passaging culture in YNB-glucose medium at an OTR_{max} of 0.012 mol l^{-1} h^{-1} (A) and 0.042 mol l^{-1} h^{-1} (B) in 250-ml flasks (shaking diameter 50 mm, shaking frequency 240 rpm, filling volumes: A: 55 ml, B: 10 ml). OTR (■) and CTR (□) were measured on-line as described in [26]. Additional Erlenmeyer flasks were used for off-line analysis of glucose (○), ethanol (▽), acetic acid (△), CDW (◇) and pH (*).

3.2. Passaging and stabilisation

During the passaging procedure the transformants are subject to a selection pressure determined by the ambient conditions. Consequently these conditions are crucial for the properties of the resulting strains. Each of the 24 transformants was passaged in eight consecutive test tube cultures in YNB-glucose medium under limited or non-limited oxygen supply, to examine its influence on the cultures (Fig. 1A).

Fig. 3A depicts the OTR, CTR, glucose, ethanol, acetic acid, cell dry weight (CDW) and pH of a representative passaging culture in YNB-glucose medium at limited oxygen supply (OTR_{max} = 0.012 mol l^{-1} h^{-1}). Initially, OTR increased exponentially, correlated with exponential growth. This was paralleled by a simultaneous CTR increase, yielding a respiratory quotient (RQ) of about 1, indicative for an aerobic turnover of glucose. When OTR_{max} was reached at 8 h, OTR remained constant, limiting the respiration to this level. CTR continuously increased up to 0.03 mol l^{-1} h^{-1}, leading to a maximal RQ of 2.7, a clear indication of a partially anaerobic metabolism. When glucose was depleted at 18 h (Fig. 3A, open circles), the CTR dropped to 0.005 mol l^{-1} h^{-1} below the OTR, which corresponded to an RQ of between 0.7 and 0.4. This referred to the aerobic turnover of a reduced carbon source. The drop of CTR was accompanied by an interim drop of the OTR to 0.008 mol l^{-1} h^{-1}. At 26 h, OTR increased to OTR_{max} again. At 35 h, OTR and CTR totally went down, indicating the depletion of all carbon sources.

The HPLC analysis provided additional information. During the phase of partially anaerobic metabolism ($RQ \gg 1$), between 8 and 18 h, 4.1 g ethanol l^{-1} (Fig. 3A, open-down triangles) and 0.8 acetic acid g l^{-1} (Fig. 3A, open-up triangles) were formed as anaerobic by-products. Biomass reached 3.5 g CDW l^{-1} (Fig. 3A, open rhombi). During the interim drop of the OTR, at 18–26 h, the culture seemed to adapt to the conversion of the reduced carbon source ethanol. In this phase 0.8 g acetic acid l^{-1} was preferably metabolised without biomass formation, obviously exclusively maintaining respiration. Concomitantly only 0.2 g ethanol l^{-1} was consumed. Thereafter 3.9 g ethanol l^{-1} was aerobically metabolised, corresponding to the RQ of significantly lower than 1. The ethanol consumption was accompanied by biomass forma-

tion, resulting in 5.4 g CDW l^{-1}. When ethanol was depleted OTR and CTR dropped, indicating the stationary phase of the culture.

The molar concentrations of consumed oxygen and evolved carbon dioxide were calculated from the OTR and the CTR integrals over fermentation time. These values were used for the stoichiometric balancing of the cultures according to Atkinson and Mavituna [45]. For this purpose, the elemental mass in the substrates was compared to the elemental mass in the products. The elemental composition of the yeast *Candida utilis* [45] served for the calculation of the element amounts in the CDW. Eqs. 2–4 show the reactions during the different culture phases of Fig. 3A per litre of culture volume. Eq. 2 shows the phase of partially anaerobic metabolism up to 18 h:

19.95 g glucose + 0.12 mol oxygen → 3.5 g CDW

+4.1 g ethanol + 0.8 g acetic acid

+0.22 mol carbon dioxide (2)

The transition phase, exhibiting maintenance respiration without growth on acetic acid between 18 and 26 h, is given by Eq. 3:

0.8 g acetic acid + 0.2 g ethanol + 0.04 mol oxygen

→ 0.0 g CDW + 0.04 mol carbon dioxide (3)

The phase of aerobic conversion of ethanol between hour 26 and hour 35 is shown by Eq. 4:

3.9 g ethanol + 0.15 mol oxygen → 2.0 g CDW

+0.09 mol carbon dioxide (4)

The substrates and products of the transition phase (Eq. 3) and subsequent aerobic metabolism on ethanol (Eq. 4) were well-balanced. But the phase of partially anaerobic metabolism (0–18 h) exhibited a conspicuous gap in the balance (Eq. 2). Product amounts corresponding to 3 g glucose equivalents l^{-1} were missing. HPLC chromatograms of the culture broth exhibited no peaks that could be referred to an additional product. Acetaldehyde, a product which can result from ethanol oxidation by *H. polymorpha* [46], could not be detected either.

Fig. 3B shows the OTR, CTR, glucose, ethanol, acetic acid, CDW and pH of a representative passaging culture in YNB-glucose medium at non-limited oxygen supply. The adjusted operating conditions ensured an OTR up to an OTR_{max} of 0.042 mol l^{-1} h^{-1} (Table 1). Initially, OTR increased exponentially until a value of 0.026 mol l^{-1} h^{-1} (Fig. 3B, filled squares), far below the OTR_{max}. CTR was slightly higher and increased concomitantly with the OTR, resulting in an RQ of about 1.2 (Fig. 3B, open squares). This corresponded to a growth-coupled aerobic consumption of glucose (Fig. 3B, open circles). At 15 h the exponential increase of the transfer rate was terminated and transfer rates decreased until 24 h. Finally, the transfer rates totally dropped down, indicating the depletion of glucose. At the time the transfer rates started to decrease, only 0.6 g ethanol l^{-1} was formed, which was then metabolised (Fig. 3B, open-down triangles). In contrast, acetic acid formation started at 15 h, reaching 2.1 g acetic acid l^{-1}. Accumulation of acetic acid was accompanied by a halt of biomass formation, reaching a maximum of 3.5 g CDW l^{-1}. pH fell from 4.0 to 2.9 during cultivation.

Apparently, acetic acid formation in *H. polymorpha* was similar to that described for the well-characterised overflow metabolism in *E. coli*. When *E. coli* is grown under aerobic conditions, acetic acid is typically formed at high glucose uptake rates. It has been suggested that the respiratory system, where NADH is re-oxidised, has a limited capacity. When respiration is saturated, the flux of acetyl-CoA is re-directed to acetate, via acetylphosphate, instead of entering the tricarboxylic acid cycle. Thus accumulation of NADH is avoided [47,48].

The decrease of transfer rates between 15 and 24 h indicated an inhibition of the culture, which was most likely due to the acidic pH values and/or the formation of acetic acid (Fig. 3B). Van Zyl et al. [49] have reported a strong acetic acid inhibition for the xylolytic yeast *Pichia stipitis*. *P. stipitis* exposed to 2 g acetic acid l^{-1} was found to exhibit lower growth rates and to produce only 25% of the ethanol formed without acetic acid exposure. The inhibitory effect increased when pH was lowered. At low pH values the amount of undissociated acetic acid increases. Undissociated acetic acid can be taken up into the cells by passive diffusion, thereby lowering the cytoplasmic pH [50]. Thus, the inhibitory effect of acetic acid probably was enhanced by the low pH values in the *H. polymorpha* cultures (Fig. 3B). The diversion of energy from growth to the maintenance of pH was found to be an important cause of acetic acid-induced growth inhibition in *S. cerevisiae* [50]. This may explain the early halt of biomass formation at the beginning of acetic acid accumulation at 15 h in the *H. polymorpha* cultures (Fig. 3B). Biomass reached only 3.5 g CDW l^{-1}, compared to 5.4 g CDW l^{-1} in oxygen-limited cultures (Fig. 3A). Accordingly, also no biomass was formed either in the presence of acetic acid during the transition phase of the oxygen-limited culture (Fig. 3A).

The low pH may have had an inhibitory effect independent of acetic acid. *H. polymorpha* grows optimally at pH values ranging from 3.0 to 6.5. It could be shown that beyond this range growth rates decreased rapidly (unpublished results). This finding is corroborated by the observation that transfer rates decreased at pH values lower than 3.0 (Fig. 3B, 15–24 h). Low pH values led to similar OTR courses in glycerol cultures which lack acetic acid formation (unpublished results). A slight acetic acid and/or pH inhibition can also be presumed in the oxygen-limited culture of Fig. 3A. The CTR decreased in this culture

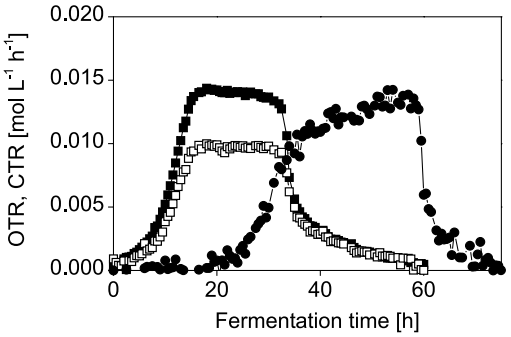

Fig. 4. Screening cultures in YNB-glycerol medium at an OTR_{max} of 0.012 mol l^{-1} h^{-1} (filling volume 55 ml, ■, □) and 0.042 mol l^{-1} h^{-1} (filling volume 10 ml, ●) in 250-ml flasks (shaking diameter 50 mm, shaking frequency 240 rpm). OTR (■, ●) and CTR (□) were measured on-line as described in [26]. The measurements were reproducible among the different transformants.

at 13 h, just at the time the acetic acid concentration increased and pH decreased to a value of 3.0.

Although the oxygen limitation led to cultures with a complex metabolism and a strong formation of ethanol, culture vitality was obviously not impaired (Fig. 3A). Consequently, the introduction of a defined oxygen limitation of the *H. polymorpha* glucose cultures may serve as an efficient strategy for the prevention of an overflow of acetic acid and its adverse effects under conditions of non-limited oxygen supply (Fig. 3B).

3.3. Screening

A range of 24 mitotically stable strains obtained after passaging were examined for phytase secretion (Fig. 1C). Phytase production was assessed in YNB-glycerol cultures under conditions of both limited oxygen supply and non-limited oxygen supply. Preculturing was carried out in YNB-glucose medium under conditions of oxygen supply identical to those in the main culture. Fig. 4 shows representative OTR profiles of the screening cultures. Under conditions of limited oxygen supply (Fig. 4, filled squares) OTR increased exponentially until an OTR_{max} of 0.014 mol l^{-1} h^{-1} was reached. This value was slightly above the calculated OTR_{max} of 0.012 mol l^{-1} h^{-1} (Table 1). Finally OTR dropped, indicating the depletion of glycerol. During the whole cultivation CTR (Fig. 4, open squares) proceeded in proportion with OTR, yielding a stable RQ of 0.75. This value stoichiometrically corresponded to the aerobic turnover of glycerol under biomass formation. Although phases of non-limited and limited oxygen supply were passed, metabolism obviously was not affected, as reflected by the stable RQ. Thus, non-fermentable glycerol provided a stable aerobic metabolism irrespective of oxygen supply. Under conditions of completely non-limited oxygen supply OTR sigmoidally increased to 0.015 mol l^{-1} h^{-1} (Fig. 4, filled circles), although an OTR_{max} of 0.042 mol l^{-1} h^{-1} was ensured (Table 1). Cultures exhibited lag phases of about 20 h.

Use of the non-fermentable carbon source glycerol was unlikely to be the reason for the conspicuous OTR profile under conditions of non-limited oxygen supply (Fig. 4, filled circles). Total oxygen consumption of 0.34 mol oxygen l^{-1} was observed with both limited and non-limited oxygen supply, indicating stable metabolic relations between oxygen and glycerol. Additionally, in both cases no by-products could be detected and maximum specific growth rates μ_{max} were 0.23 h^{-1}.

The screening precultures were conducted in YNB-glucose medium under the same conditions of limited or non-limited oxygen supply as the screening main cultures (Fig. 5). Thus, the same oxygen-dependent metabolism was induced in the precultures (Fig. 5) as already explained for passaging cultures (Fig. 3). According to findings of Grauslund et al. [51,52] for *S. cerevisiae*, the promoters of genes encoding glycerol-catabolising enzymes are derepressed by ethanol. Thus, inocula taken from oxygen-limited glucose precultures were presumably adapted to glycerol utilisation in the screening main cultures and lag phases were prevented (Fig. 5A). Precultures under conditions of non-limited oxygen supply metabolised glucose and produced acetic acid as a by-product (Fig. 5B). According to Grauslund et al. [51,52] glucose represses genes encoding glycerol-catabolising enzymes. Hence, these enzymes had to be synthesised prior to glycerol utilisation in the screening cultures, most likely causing extended lag phases (Fig. 5B). Moreover, sigmoidal OTR profiles, similar to those found for the screening cultures with non-limited oxygen supply (Fig. 4, filled circles), frequently

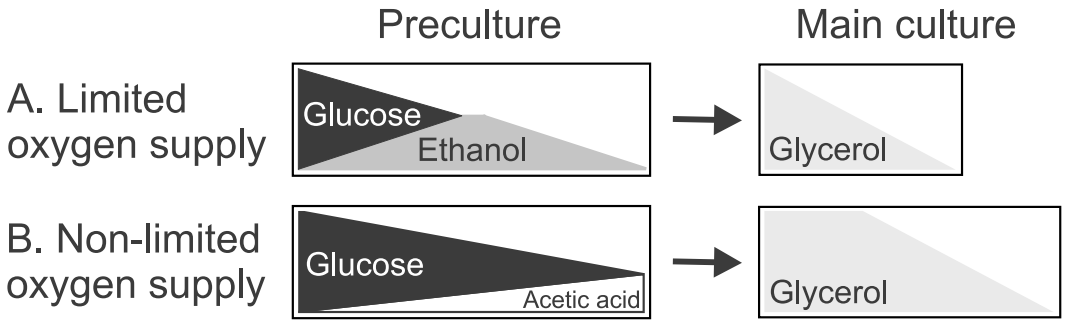

Fig. 5. Schematic diagram of the consumption of carbon sources and formation of by-products in the screening precultures (YNB-glucose medium) and the screening main cultures (YNB-glycerol medium) under limited oxygen supply (A) and non-limited oxygen supply (B).

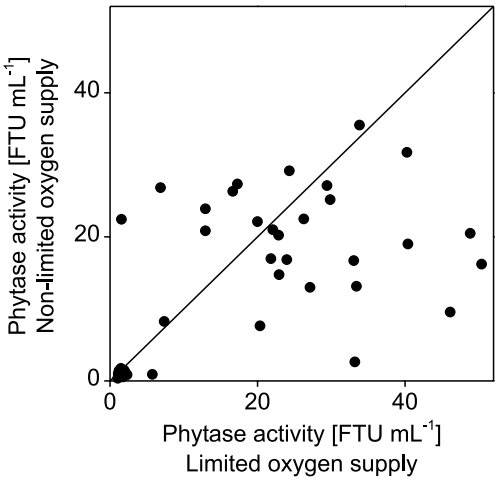

Fig. 6. Phytase activities during screening and after passaging in test tube cultures under non-limited (OTR_{max} = 0.081 mol l^{-1} h^{-1}) and limited oxygen supply (OTR_{max} = 0.012 mol l^{-1} h^{-1}) for each of the 24 transformants at the passaging stages 1, 3, 5 and 8.

indicate inhibitions [53]. Thus, the screening cultures with non-limited oxygen supply seemed to be affected by an inhibition, possibly arising from overflow metabolism and acetic acid formation in the preculture (Fig. 5B). In this context, the oxygen-limited precultivation was beneficial, providing vital growth of the subsequent screening main culture (Fig. 4, filled squares).

Phytase activities of 24 transformants (Fig. 1C) were measured after cultivation in test tubes with YNB-glycerol medium. Cell extracts exhibited only negligible phytase activities, demonstrating the efficient secretion of the enzyme (data not shown). The cultures were conducted under conditions of limited or non-limited oxygen supply as characterised before (Fig. 4). Half of the investigated transformants exhibited almost no phytase activity (below 2 FTU ml^{-1} over all examined stages of passaging) (Fig. 6). This was probably due to the integration of defective expression vectors lacking a functional phytase gene. Strain development can be more efficient when such candidates are selected out at an early stage of passaging. Ten of the phytase-producing transformants (>2 FTU ml^{-1}) exhibited a constant high phytase activity during the passaging stages (data not shown), two other strains showed increasing phytase secretion.

Phytase production was analysed in relation to the different conditions of oxygen supply. Fig. 6 compares the phytase activities during screening and after passaging in test tube cultures under non-limited or limited oxygen supply for each of the 24 transformants at the examined passaging stages. Phytase activity was on average 25% higher when transformants were cultivated under limited oxygen supply. As can be seen in the parity plot, two thirds of the phytase-producing transformants revealed higher phytase activities when passaged and screened under limited oxygen supply. Higher phytase activity under limited oxygen supply was not stringent. One third of the transformants exhibited a higher phytase activity under non-limited conditions. Because of these results, no stringent mechanistic model can be deduced. The oxygen-deprived state or the alternating utilisation of glucose and ethanol during oxygen-limited passaging (Figs. 1A and 3A) possibly facilitated the amplification and integration of the expression vector. Thus phytase synthesis was possibly enhanced due to a gene dosage effect. In previous studies of production strains, phytase production could be maximised by increasing the gene dosage from 60 to 120 copies by supertransformation (unpublished results). On the other hand, the integration of expression vectors may have been impaired by the obvious inhibition of the passaging cultures under non-limited oxygen supply (Fig. 3B). Additionally, the long lag phases and the apparent inhibition under non-limited oxygen supply (Fig. 4, filled circles) presumably resulted in a reduced heterologous gene expression. The *FMD* promoter driving heterologous gene expression could also be under negative oxygen control.

In contrast to the beneficial effect of oxygen limitation on phytase production presented here, reduced phytase formation of *H. polymorpha* was found when oxygen was limited in a fed-batch process on glucose, as reported by Mayer et al. [6]. Moreover, this process yielded extremely high concentrations of 13.5 g phytase l^{-1} under conditions of non-limited oxygen supply. The high performance of this process was likely due to the limited feed of glucose, preventing overflow metabolism and its adverse effects.

4. Conclusions

Fed-batch production processes are usually performed under conditions of non-limited oxygen supply to ensure maximal productivity. In these processes overflow metabolism is usually prevented by a limited carbon feed. For the high-throughput passaging and screening presented here, cultivations were carried out on a small test tube scale in batch mode, thus minimising the operating expenses. As a consequence, an overflow metabolism during passaging and screening induced by excessive glucose supply under non-limited oxygen conditions led to a reduced phytase production. In this case, the introduction of controlled oxygen limitation is a superior method, because the metabolic flux can be channelled from overflow metabolism to a less harmful anaerobic by-product formation. It is thus of great significance for bioprocess development to identify the conditions of oxygen supply and to analyse their effects, thus ensuring an optimal adjustment of aeration conditions. The characterisation of hydrodynamics and OTR_{max} in test tubes as well as the on-line measurement of OTR and CTR in the passaging and screening cultures present a rational approach for the analysis of

oxygen supply and its influence during bioprocess development.

Nomenclature

a	specific mass transfer area (m^{-1})
CDW	cell dry weight (g l^{-1})
CTR	carbon dioxide transfer rate (mol l^{-1} h^{-1})
D_{O2}	oxygen diffusion coefficient (m^2 h^{-1})
FTU	phytase activity, enzyme amount releasing 1 μmol of inorganic phosphate per min from sodium phytate at pH 5.5 and 37°C
K	correlation factor, quotient of the OTR_{max} of two different liquid systems (dimensionless)
L_{O2}	oxygen solubility (mol l^{-1} bar^{-1})
MLH	relative maximum liquid height (mm)
OTR	oxygen transfer rate (mol l^{-1} h^{-1})
OTR_{max}	maximum oxygen transfer capacity (mol l^{-1} h^{-1})
$OTR_{max} \cdot V_L$	oxygen transfer velocity (mmol h^{-1})
RQ	respiratory quotient (dimensionless)
V_L	liquid volume (ml)
μ_{max}	maximum specific growth rate (h^{-1})

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