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Research review paper

Cultivation strategies to enhance productivity of *Pichia pastoris*: A review

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ABSTRACT

Pichia pastoris, a methylotrophic yeast, is an established system for the production of heterologous proteins, particularly biopharmaceuticals and industrial enzymes. To maximise and optimise the production of recombinant products, recent molecular research has focused on numerous issues including the design of expression vectors, optimisation of gene copy number, co-expression of secretory proteins such as chaperones, engineering of glycosylation and secretory pathways, *etc.* However, the physiological effects of different cultivation strategies are often difficult to separate from the molecular effects of the gene construct (*e.g.*, cellular stress through over-expression or incorrect post-translational processing). Hence, overall system optimisation is difficult, even though it is urgently required in order to describe and understand the behaviour of new molecular constructs. This review focuses on particular aspects of recombinant protein production related to variations in biomass growth and their implications for strain design and screening, as well as on the concept of rational comparisons between cultivation systems for the development of specific production processes in bioreactors. The relationship between specific formation rates of secreted recombinant proteins, q_p, and specific growth rates, μ, has been analysed in a conceptual attempt to compare different systems, particularly those based on AOX1/methanol and GAP/glucose, and this has now evolved into a pivotal concept for bioprocess engineering of *P. pastoris*.

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1. Introduction

As a production system, the methylotrophic yeast *Pichia pastoris* is distinguished by very high biomass concentrations, its potential to produce large quantities of correctly processed recombinant proteins (intracellularly or secreted) of either prokaryotic or eukaryotic origins, and containing a tightly regulated AOX1-promoter (Cereghino and Cregg, 2000). Extensive lists of proteins that have been successfully produced with *P. pastoris* highlight the increasing future relevance of this microbial production system (reviewed in Ahmad et al., 2014; Calik et al., 2015; Cereghino and Cregg, 2000; Li et al., 2007; Macauley-Patrick et al., 2005). Due to heightened interest in its application, new genetic tools for *P. pastoris* have been developed (Ahmad et al., 2014; Delic et al., 2014; Vogl and Glieder, 2013; Vogl et al., 2013), and thus the availability of new strains has intensified the need for a systematic design of cultivation and production processes with this yeast (Buchetics et al., 2011; Potgieter et al., 2010; Rebnegger et al., 2014).

Existing shortcomings of the *P. pastoris* production system are addressed either by genetic strain engineering (Idiris et al., 2010; Puxbaum et al., 2015) or at the level of the cultivation process in bioreactors. Generally, bioprocess design aims at identifying optimum conditions for biomass growth and product formation, including pH, temperature, oxygen and nutrient supply (*e.g.*, Calik et al., 2015; Cos et al., 2006; Jahic et al., 2006). Central to identifying the physiological constraints of *P. pastoris* is the development of a suitable mode of adding carbon and energy substrates, *i.e.*, the feed profile or feeding strategy (Jahic et al., 2006; Khatri and Hoffmann, 2006b; Maurer et al., 2006; Spadiut et al., 2014a; Zhang et al., 2007). Appropriate culture conditions and feed strategies vary, depending on whether recombinant proteins are produced under the control of the inducible AOX1-promoter in Mut^+ or Mut^S *P. pastoris* strains, or the constitutive GAP-promoter (*e.g.*, Calik et al., 2015; Maurer et al., 2006; Potgieter et al., 2010; Yamawaki et al., 2007).

Available cultivation protocols are mainly based on those described in the commercial Invitrogen expression kit (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006). However, a recent trend is to move away from standard protocols (*i.e.*, fixed recipes for growth-dependent and growth-independent production kinetics) towards a conceptual approach that allows for the development of a specific process strategy that is tailored to both a particular product/genetic construct combination and the characteristics of specific bioreactor equipment (Maurer et al., 2006; Spadiut and Herwig, 2014; Zhang et al., 2007). However, the concept itself should be generally applicable and thus transferable between different strains and/or equipment.

As manufacturing processes aim at producing a maximum amount of product with given quality specifications and in the minimum process time (*i.e.*, space–time-yield or volumetric productivity), specific productivity q_p, maximum biomass $x \cdot V$, and productive time (*i.e.*, time between induction of product formation and harvest) are critical influencing factors. In line with its aim of describing a pivotal concept in bioprocess engineering, this review sharply focuses on the relationship between biomass growth (expressed in terms of specific growth rate μ) and product formation (expressed as specific productivity q_p), and validates this concept for process development with real experimental data from *P. pastoris* processes. The advantage of the concept presented in this review is using values of μ and q_p that are strain specific and thus comparable between different systems (*i.e.*, strain designs and bioreactors), and are independent of process-specific settings such as biomass concentration, reactor volume or process time. In contrast, titres and rates of feed addition, which are typically published for process comparison reasons, are not directly comparable because of their interdependencies.

This review begins by explaining the meaning and use of the physiological characteristics of biomass growth and recombinant protein formation, as well as their implications for reducing the number of clones during screening in batch mode. It further exemplifies formation kinetics (*i.e.*, the $q_p(\mu)$-relationships) published in the literature for several recombinant proteins that are produced under the control of AOX1- and GAP-promoters. The rationale behind a customisable process strategy, starting with the physiological characterisation of a particular strain, is that it can be logically adapted to any particular recombinant strain and/or stirred bioreactor, and helps us to understand the difference between this and standard protocols. Finally, information compiled from the literature is translated into recommendations on best practice, and future innovative development is depicted.

Recent literature claims a lack of strategies and workflows for systematic bioprocess development, which is required to shorten development times and for increasing productivities (Knepper et al., 2014; Posch and Herwig, 2014). This review therefore goes beyond standard cultivation recipes towards concepts that enable an appropriate process strategy to be rationally adapted to the particular features of a specific recombinant strain, *i.e.*, combinations of host, promoter and substrate, product and gene copy number, as well as the individual characteristics of the bioreactor equipment used. The information assembled takes a process-oriented perspective that is not yet available in such focussed depth in one review, and is reinforced by several other excellent general overview articles on *P. pastoris* technology (Ahmad et al., 2014; Cos et al., 2006; Delic et al., 2014; Gasser et al., 2008; Hartner and Glieder, 2006; Idiris et al., 2010; Jahic et al., 2006; Mattanovich et al., 2012, 2014; Spadiut et al., 2014a; Vogl and Glieder, 2013; Zhang et al., 2009).

2. Kinetics of biomass growth and recombinant protein formation

In general, two distinguishable operational strategies for *P. pastoris* processes are derived from the most widely used promoters: inducible, strong AOX1 (*a*lcohol *ox*idase 1) or constitutive GAP (*g*lycer*a*ldehyde-3-*p*hosphate dehydrogenase). The major disadvantage of the mostly favoured AOX1 over GAP is that it requires methanol for recombinant protein production. Thus, methanol, added as a single carbon and energy source and metabolised by *P. pastoris* cells, is principally required both for induction of product formation and growth to produce biomass (NB. In contrast to current *Pichia* systems, the well-established *E. coli* system, typically induced by IPTG, offers an advantage in controlling induction and growth by two independent principles: by separately controlling growth by addition of the C-substrate, and production by addition of the IPTG-inducer).

Since genetic strain engineering (and overproduction of a heterologous protein) can considerably affect growth performance with any (recombinant) *P. pastoris* strain, determinations of the key stoichiometric and kinetic characteristics of biomass growth and product formation are the basis for the rational design of a production process in a bioreactor. Thus, for each combination of genetic construction (*i.e.*, mainly determined by the promotor used and type of heterologous protein) and culture conditions (*i.e.*, substrate utilised, pH, T, *etc.*) an optimum

specific growth rate (μ_{opt}) can be determined where production performance is best. With AOX1-strains, this μ_{opt}, sought-after during process development, typically lies at a certain value between conditions of no-growth and maximum possible growth, μ_{max}.

2.1. Biomass growth

Wild-type *P. pastoris* is able to grow on many different carbon and energy sources (Inan and Meagher, 2001), of which glycerol, glucose and methanol are the most commonly used in manufacturing processes. The choice of carbon-substrate and, therefore, the feasible operational range with respect to specific growth rate (μ) and optimum productivity (q_p) is dependent on the chosen promoter. In addition, values of the growth characteristics (*i.e.*, μ_{max}, $Y_{x/s}$, m_s, see nomenclature given in Table 1) are crucial since they give a direct indication of whether the strain is physiologically impaired due to the introduction and expression of a foreign gene (Cos et al., 2006; Heyland et al., 2011; Hyka et al., 2010). Heavily impaired strains tend to stop growing, die and lyse, which causes problems in bioreactor operation because of heavy foaming as well as impaired product quality due to the release of host proteins or intracellular proteases (Jahic et al., 2003, 2006; Zhou and Zhang, 2002).

2.1.1. Phenotypes with respect to methanol utilisation

Strains expressing a heterologous gene under the control of the AOX1-promoter utilise methanol, which is required both as an inducer, and for biomass growth and production (Hartner et al., 2008). The oxidation of methanol by alcohol oxidase is encoded by two genes, *AOX1* and *AOX2* (Cregg et al., 1989). Depending on whether one or other of those genes is knocked out, three *P. pastoris* phenotypes are distinguishable (Cos et al., 2006): strains comprising functional versions of both genes (Mut$^+$, methanol utilising plus), mutants with an inactivated *AOX1* gene (Mut^s, methanol utilising slow) and mutants lacking both *AOX* genes (Mut$^-$, methanol utilising minus). Integration of a pAOX1-based expression cassette into the *P. pastoris* genome at the *AOX1* locus allows either integration into or replacement of the *AOX1*-gene, resulting in a functional (Mut$^+$ phenotype) or a dysfunctional *AOX1* gene (Mut^s phenotype). Mut$^-$ strains, however, are not able to metabolise methanol, which then only acts as an inducing agent for recombinant protein production (Chiruvolu et al., 1997). A commercially available strain, MC100-3 (*his4 arg4 aox1Δ:SARG4 aox2Δ:Phis4*), has both *AOX* genes deleted but retains the ability to induce recombinant protein production at high levels using the AOX1-promoter (Cregg and Madden, 1989). Such a strain represents the first example of decoupling growth and protein production during the same cultivation phase, since co-feeding with a second carbon source, such as glucose, is necessary (*i.e.*, in addition to a certain concentration of methanol that is not metabolised and acts only as an inducer; Chiruvolu et al., 1997). However, more recent literature reveals that these strains are, for non-disclosed reasons, not currently in use. A further problem with such a system can be the need to monitor (and continuously adjust) the methanol concentration as it is stripped from the culture medium.

With excess methanol as a substrate, at 30 °C, Mut$^+$ strains achieved maximum specific growth rates of up to 0.15 h^{-1} (Kobayashi et al., 2000). For Mut^S strains, typical μ_{max}-values were considerably lower, *e.g.*, up to 0.035 h^{-1} (Brierley et al., 1990). Commonly observed effects of μ_{max} values decreasing at cultivation temperatures below 30 °C, depending on the composition of culture medium, also apply to *P. pastoris* (Curvers et al., 2001b; Kovarova et al., 1996). A particular strain's ability to achieve a given μ_{max} value has different relevance in batch *versus* fedbatch culture modes. During a fedbatch process, μ is controlled at its optimum with respect to growth or production. Thus, under well-controlled fedbatch conditions, the same (low) μ-value can be set for either Mut^S or Mut$^+$ strains. The essential benefit of working with a Mut^S strain is that, in the case of methanol accumulation, this strain does not accelerate its growth as fast as Mut$^+$ would do, and hence large scale manufacturing with Mut^S may be easier (Cos et al., 2005; Stratton et al., 1998).

2.1.2. Relation of biomass growth and heterologous protein production

During batch cultivation, in which substrate is available in excess, biomass growth is unrestricted and growth characteristics can be determined directly (Table 2, Fig. 1). With glycerol or glucose, *P. pastoris* grows significantly faster (*i.e.*, 1.7–8.5 times faster for the strains given in Table 2) than with methanol. Typical diauxic behaviour is observed in batch cultures (*i.e.*, glucose or glycerol repress the utilisation of methanol and these substrates are therefore used sequentially) (Jungo et al., 2007). Strains engineered for recombinant production of a heterologous protein often exhibit maximum specific growth rates (Table 2) significantly lower than those observed with a non-engineered host strain. Recombinant Mut$^+$ strains are reported to grow in excess methanol at a wide range of μ from 0.028 h^{-1} to 0.154 h^{-1}, and Mut^S strains at 0.011 h^{-1} to 0.035 h^{-1}. Decreased maximum specific growth rates are most probably related to genetic burdens introduced by strain engineering and/or metabolic burdens of recombinant protein production (Gasser et al., 2008; Glick, 1995; Heyland et al., 2011). During batch growth with glucose, μ_{max} varied from 0.28 h^{-1} to 0.16 h^{-1} for strains differing with respect to productivity, whereas the best-producer strains grew slower (Heyland et al., 2011). Furthermore the specific glucose uptake rate (q_s) did not change in accordance with the specific growth rate, and TCA cycle activity was found to be constant irrespective of significantly reduced growth rates. This data suggests that the additional resources required for recombinant protein production resulted in reduced specific growth rates (Heyland et al., 2011). Regarding strain design, high levels of expression of a protein may also have a detrimental effect on cell metabolism, but this might be better tolerated by the host at low gene dosage; hence, the assumption that 'higher gene dosage equals higher production' is not necessarily true (Aw and Polizzi, 2013). For instance, Mut$^+$ strains with more than 12 recombinant gene copies had lower substrate utilisation and specific growth rates (0.01 h^{-1} to 0.02 h^{-1}) than strains with fewer gene copies (0.025 h^{-1} to 0.029 h^{-1}) (Zhu et al., 2009). A similar effect was not observed in Mut^S strains: both single- and multi-copy Mut^S strains exhibited comparable growth rates while Mut$^+$ single-copy strains grew faster than Mut$^+$ multi-copy strains (Table 2) (Cos et al., 2005).

Table 1
Nomenclature.

Abbreviation	Unit	Description
c_p	mg l^{-1}	Product titre, concentration of product (in supernatant)
$c_p \cdot V_s$	mg	Amount of product
CPR	mol h^{-1}	Carbon dioxide production rate
F	g h^{-1}	Feed rate
F_0	g h^{-1}	Initial feed rate
m_s	g g^{-1} h^{-1}	Specific maintenance rate
μ	h^{-1}	Specific growth rate
μ_{max}	h^{-1}	Maximum specific growth rate
q_p	mg g^{-1} h^{-1}	Specific productivity (specific rate of product formation)
$q_{p,max}$	mg g^{-1} h^{-1}	Maximum specific productivity (specific rate of product formation)
q_s	g g^{-1} h^{-1}	Specific rate of substrate utilisation
$q_{s,max}$	g g^{-1} h^{-1}	Maximum specific substrate utilisation rate
r_p	mg l^{-1} h^{-1}	Volumetric productivity (space–time-yield)
s	g l^{-1}	(Residual) concentration of substrate in the reactor
s_0	g l^{-1}	Initial substrate concentration
t	h	Process time
V	l	Working volume
V_0	l	Initial working volume
V_s	l	Volume of supernatant
w_{in}	g g^{-1}	Mass fraction of substrate in the feed solution
x	g l^{-1}	Concentration of biomass
$x \cdot V$	g	Mass of biomass
x_0	g l^{-1}	Initial biomass concentration
$Y_{p/x}$	mg g^{-1}	Observed yield (product/biomass)
$Y_{x/s}$	g g^{-1}	Observed yield (biomass/substrate)
$Y_{x/s,max}$	g g^{-1}	Maximum yield (biomass/substrate)

Table 2
Kinetic and stoichiometric characteristics of *Pichia pastoris* Mut^+ and Mut^s strains.
If not otherwise stated, values are typically based on cultivation of recombinant strains at 30 °C with the listed carbon sources. The table entries are listed in the order of decreasing maximum specific growth rates (μ_{max}) achieved with the inducing substrate (methanol) while producing a recombinant protein (marked in bold). Data listed for glycerol relate to biomass growth without product formation. $Y_{x/s}$ (biomass to substrate yield) and m_s (specific maintenance rate) are based on cell dry weight in grams.

	Methanol			Glycerol			Heterologous product (origin)	Gene copy number	Strain	Reference
	μ_{max} (h^{-1})	$Y_{x/s}$ ($g\,g^{-1}$)	m_s ($g\,g^{-1}\,h^{-1}$)	μ_{max} (h^{-1})	$Y_{x/s}$ ($g\,g^{-1}$)	m_s ($g\,g^{-1}\,h^{-1}$)				
Mut^+	**0.154**	0.38	0.023				Serum albumin (human)	2	GS115	Kobayashi et al. (2000)
	0.14						Lysozyme (bovine)	NA	GS115	Brierley et al. (1990)
	0.14	0.42	0.014	0.24	0.61	0.009	Avidin	NA	GS115	Jungo et al. (2006)
	0.12	0.41		0.28[a]	0.72[a]		Trypsinogen (pocine)	NA	X-33	Paulova et al. (2012)
	0.101	0.38	0.011		0.45		Insulin precursor (porcine)	6 to 8	GS115	Hang et al. (2008)
	0.10–0.13	0.15		0.20–0.29	0.45–0.47		Serum albumin (human)	NA	GS115	Ren et al. (2003)
	0.07	0.33[b]	0.016[b]				α-Galactosidase (coffee bean)	NA	GS115	Zhang et al. (2005)
	0.051			0.18	0.5		Lipase (*Rhizopus oryzae*)	Single	X-33	Cos et al. (2005)
	0.028			0.18	0.5		Lipase (*Rhizopus oryzae*)	Multi	X-33	Cos et al. (2005)
	–	0.16	0.03	0.21	0.43	0.032	hG-CSF (human)	2	GS115	Bhatacharya et al. (2007)
Mut^S	**0.035**						Lysozyme (bovine)	NA	GS115	Brierley et al. (1990)
	0.018			0.18	0.5		Lipase (*Rhizopus oryzae*)	Multi	X-33	Cos et al. (2005)
	0.015						Lipase (*Rhizopus oryzae*)	Single	KM71	Cos et al. (2006)
	0.014			0.18	0.5		Lipase (*Rhizopus oryzae*)	Single	X-33	Cos et al. (2005)
	0.011	0.30	0.026	0.093	0.619		Mini proinsulin	Multi	GS115	Pais et al. (2003)

NA not available.
[a] Values for glucose as substrate.
[b] Published parameter adapted from wet cell weight to dry cell weight by a factor of four.

2.1.3. Recommendations for best practice in process development

In-depth analysis of published data on recombinant protein production with *P. pastoris* drives the following recommendations:

- Maximum specific growth rate is a critical factor indicating impaired physiology due to recombinant protein production (or merely introduction of the foreign gene into the strain). A careful determination of μ_{max} (using several data points for various substrates, *e.g.*, glucose, glycerol, and methanol) is therefore mandatory in commencing any process development.
- Recombinant Mut^+ strains are reported to grow in excess methanol at a wide range of μ from 0.028 h^{-1} to 0.154 h^{-1}, and Mut^S strains in a narrower range from 0.011 to 0.035 h^{-1}. Therefore, under well-controlled fedbatch conditions, the same (low) μ-value can be set (and controlled) for either Mut^S or Mut^+ strains. The further advantage of processes with Mut^S strains is their robustness with respect to overfeeding with methanol.

2.2. Production kinetics

The specific rate of biomass growth (μ) is a critical factor in enhancing product formation (Kobayashi et al., 2000; Potgieter et al., 2010; Schenk et al., 2008; Sinha et al., 2007; Zhang et al., 2000, 2005) as well as affecting product quality (Schenk et al., 2008; Wu et al., 2011). The relationship between specific productivity (q_p in mg product built per g cell dry weight and per hour, Figs. 2, 3) and μ (h^{-1}) reflects the equilibrium between various processes in a cell until the product is secreted (*i.e.*, induction of gene expression, translation, protein folding and degradation in the endoplasmic reticulum, flux of folded protein out of the ER, and trafficking through the secretory machinery). This relationship, also termed 'production kinetics' (Fig. 2), is essential for the design of production strategies in which growth is retained at a certain optimum μ-value by the controlled addition of carbon-substrate in fedbatch mode (Barrigon et al., 2015). Since broad and systematic experience in the production of recombinant enzymes with *P. pastoris* is not yet available, the $q_p(\mu)$-relationship (Fig. 2)

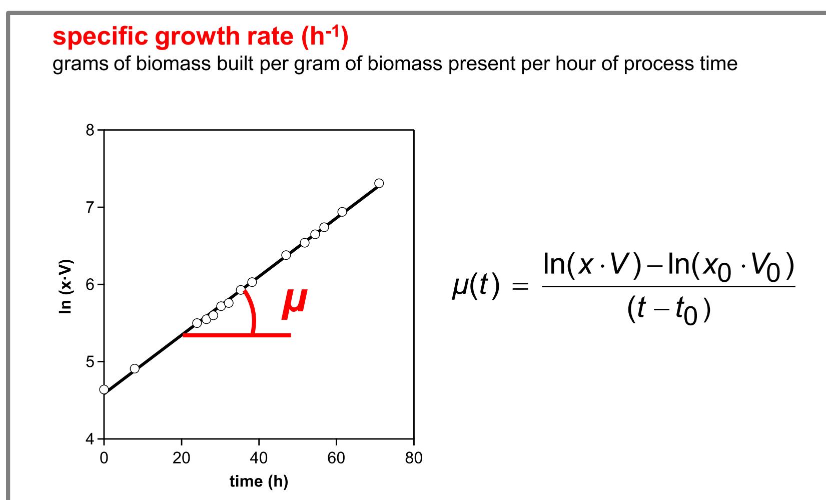

Fig. 1. Calculation box: specific growth rate (μ) and maximum specific growth rate (μ_{max}). Specific growth rate can be calculated similarly for batch and fedbatch cultivations based on the logarithm of biomass ($x \cdot V$). A constant specific growth rate of (0.038 ± 0.001) h^{-1} was determined for 71 h of fedbatch production with *Pichia pastoris* secreting *Candida antarctica* lipase B (unpublished results).

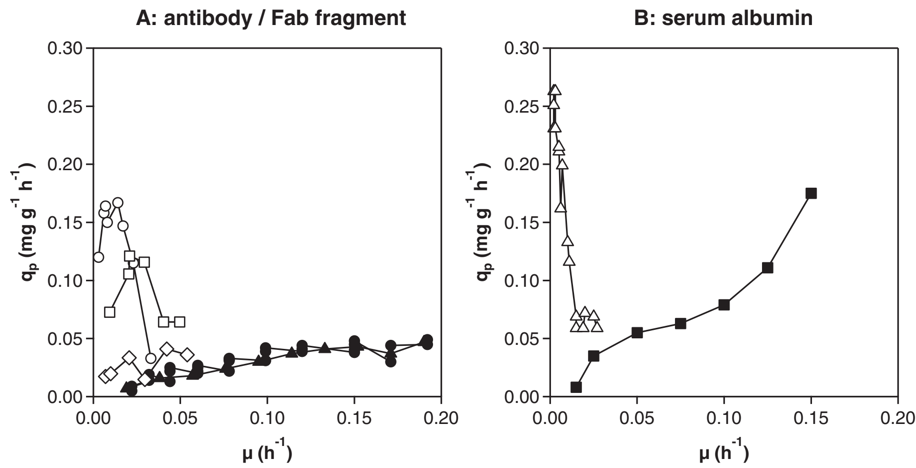

Fig. 2. Production kinetics for *AOX1* and *GAP*-regulated expression. Published kinetics of secreted antibody/Fab fragments (A) and serum albumin (B) are compared. Recombinant products under control of the AOX1-promoter and methanol (open symbols) are distinguished from GAP-promoter with glucose as a substrate (black symbols). Culture conditions are summarised in Table 2. A: (○) Potgieter et al. (2010); (◇) Yamawaki et al. (2007), 10% DO-stat; (□) Yamawaki et al. (2007), 3.9 g l^{-1} methanol-stat; (●) Maurer et al. (2006); (▲) Buchetics et al. (2011). B: (△) Kobayashi et al. (2000); (■) Rebnegger et al. (2014).

is not predictable *a priori* and has to be determined empirically (Potgieter et al., 2010). Such experimental approaches for establishing product formation kinetics are described in Section 3.2.2.

An exemplary overview of published data on optimum specific growth rates at which the highest specific productivity ($q_{p,\max}$) was reached for different strains, promoters and products, is given in Table 3. Typically, production kinetics controlled by the AOX1-promotor, utilising methanol or mixtures of substrates with methanol, were investigated for $\mu < 0.08$ h^{-1} (Curvers et al., 2001b; Zhang et al., 2005) or even $\mu < 0.03$ h^{-1} (Hang et al., 2008; Kobayashi et al., 2000; Min et al., 2010; Potgieter et al., 2010). Only two kinetic-relationships with low q_p-values (<0.03 mg l^{-1} h^{-1}) were investigated up to a μ of 0.14 h^{-1} (Jungo et al., 2006; Schenk et al., 2008). For processes in which production is controlled by the GAP-promoter, utilising glucose as a growth and energy substrate, specific growth rates of up to 0.2 h^{-1} were investigated (Khasa et al., 2007).

Generally, a multitude of relationships between production and growth can be found in the literature, *i.e.*, increasingly or decreasingly

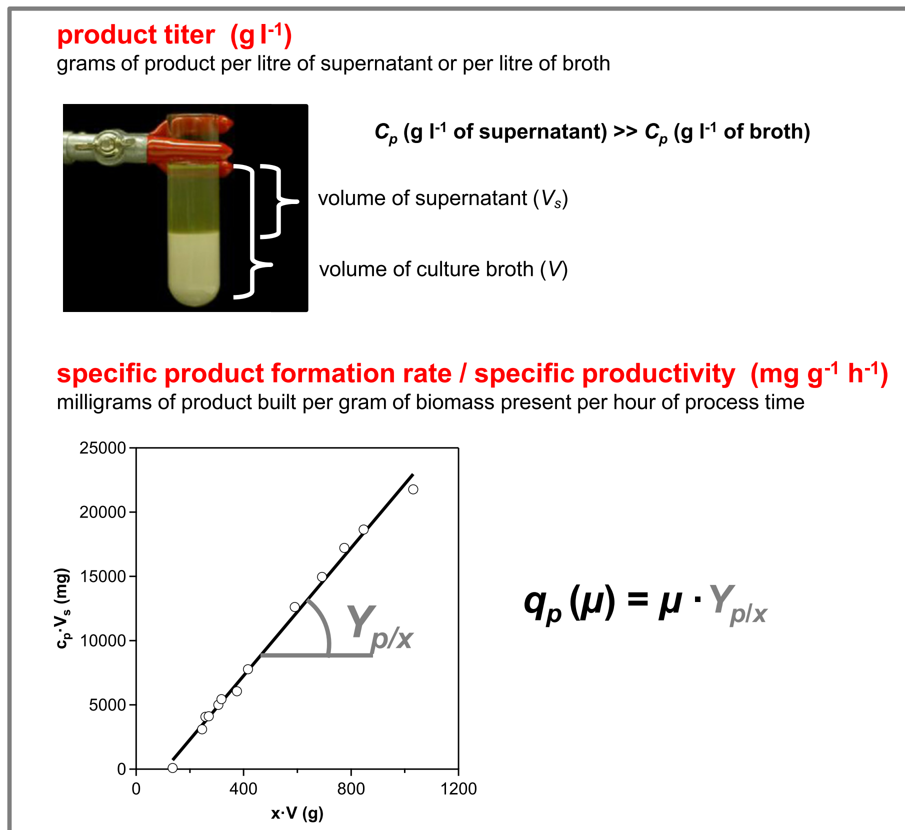

Fig. 3. Calculation box: description of product formation.

Table 3
Kinetics of recombinant secreted products.
The entries are listed in increasing order of optimal specific growth rates (μ) for recombinant product formation. Formation kinetics of antibodies and serum albumin are compared in detail in Fig. 1 (marked in bold). Maximum specific product formation rates ($q_{p,\max}$) are expressed as milligrams of product synthesised per gram of cell dry weight per hour.

Promoter	Cultivation conditions					Product formation kinetics					Reference	
	Utilisation	Medium	Temp. (°c)	pH	Mode	μ-range (h^{-1})	$\mu(q_{p,\max})$ (h^{-1})	$q_{p,\max}$ ($mg\ g^{-1}\ h^{-1}$)	Kinetics	Heterologous product (origin)		
AOX	met (Mut$^+$)	Defined	30.0	5.85	Fedbatch	0.002–0.027	**0.002**	0.26	Negatively growth related	**Serum albumin (human)**	Kobayashi et al. (2000)	Fig. 4
AOX	met (Mut$^+$)	Defined	30.0	5.0	Fedbatch	0.004–0.021	**0.004**	0.023	Negatively growth related	Saxatilin (*Gloydius saxalitis*)	Min et al. (2010)	Table 2
AOX	met (Mut$^+$)	Defined	30.0	5.0	Fedbatch	0.006–0.01	**0.01**	0.2	Growth coupled[a]	hG-CSF (human)	Bhatacharya et al. (2007)	Fig. 7
AOX	met (Mut$^+$)	Complex	24.0	6.5	Fedbatch	0.003–0.03	**0.014**	0.17[b]	Bell shaped	**Antibody[c](IgG1)**	Potgieter et al. (2010)	Fig. 3
AOX	met (Mut$^+$)	Defined	30.0	5.0	Fedbatch	0.003–0.023	**0.016**	0.1	Bell shaped	Insulin precursor (porcine)	Hang et al. (2008)	Fig. 3
AOX	met (Mut$^+$)	Defined	30.0	5.0	Continuous[d]	0.009–0.05	**0.02**	0.12	Bell shaped	**scFv fragment (anti-bisphenol A)**	Yamawaki et al. (2007)	Fig. 7
AOX	met (Mut$^+$)	Defined	30.0	5.0	Fedbatch	0.015–0.06	**0.03**	0.42[b]	Bell shaped (complex)	α-Galactosidase (coffee bean)	Zhang et al. (2005)	Fig. 3
AOX	met (Mut$^+$)	Defined	30.0	5.0	Continuous[e]	0.007–0.05	**0.04**	0.004	Linear (oxygen limited)	**scFv fragment (anti-bisphenol A)**	Yamawaki et al. (2007)	Fig. 7
AOX	met:glu[f] (Mut$^+$)	Defined	30.0	5.9	Continuous	0.03–0.20	**0.07**	0.69	Bell shaped	Trypsinogen (porcine)	Paulova et al. (2012)	Fig. 2
AOX	met (Mut$^+$)	Defined	27.5	5.0	Continuous	0.04–0.08	**0.08**	0.24	Growth coupled	Chymotrypsinogen B (human)	Curvers et al. (2001b)	Fig. 6
AOX	met:gly (Mut^S)	Defined	NA	NA	Continuous	0.01–0.09	**0.09**	0.07	Growth coupled	Antifreeze protein (sea raven)	d'Anjou and Daugulis (2001)	Fig. 2
AOX	met (Mut$^+$)	Defined	30.0	5.0	Continuous	0.03–0.12	**0.12**	0.03	Growth coupled	Avidin	Jungo et al. (2006)	Fig. 8
AOX	met (Mut$^+$)	Defined	30.0	5.0	Fedbatch	0.02–0.14	**0.14**	0.03	Growth coupled	Avidin	Schenk et al. (2008)	Fig. 2
GAP	glu	NA	25.0	5.85	Continuous	0.015–0.15	**0.15**	0.15	Growth coupled	**Serum albumin (human)**	Rebnegger et al. (2014)	Fig. 1
GAP	glu	Defined	25.0	5.0	Continuous	0.02–0.19	**0.19**	0.049	Growth coupled	**Fab fragment (anti-HIV antibody 2F5)**	Maurer et al. (2006)	Fig. 1
GAP	glu	Defined	25.0	5.0	Continuous	0.02–0.19	**0.19**	0.05	Growth coupled	**Fab fragment (human antibody 3H6)**	Buchetics et al. (2011)	Fig. 2
GAP	glu	Complex	30.0	5.0	Continuous	0.02–0.20	**0.20**	0.49	Growth coupled	hG-CSF (human)	Khasa et al. (2007)	Fig. 5

NA not available.
[a] Computed relationship.
[b] Published parameter adapted from wet cell weight to cell dry weight by factor four.
[c] Glycoengineered strain.
[d] Constant methanol concentration of 3.9 g l^{-1}.
[e] Constant dissolved oxygen at 10%.
[f] Substrate mixture of 40:60 carbon of methanol:carbon of glucose.

linear, hyperbolic or bell-shaped (*e.g.*, Bhatacharya et al., 2007; Curvers et al., 2001b; Graslund et al., 2008; Hang et al., 2008; Jungo et al., 2006; Kobayashi et al., 2000; Min et al., 2010; Potgieter et al., 2010; Schenk et al., 2008; Zhang et al., 2005). Nevertheless, for strains containing the GAP-promoter, product formation was found to increase with specific growth rates almost up to $\mu_{\max}$ (Khasa et al., 2007; Maurer et al., 2006). For AOX1 Mut$^+$ strains cultured with methanol, the optimum specific growth rate for product formation (μ_{opt} at $q_{p,\max}$) ranged from low μ-values of 0.002 h^{-1} to values up to $\mu_{\max}$ of 0.14 h^{-1} (Table 3). Processes with low optimal μ-values < 0.004 h^{-1} showed negative growth-related relationships between production and growth (Kobayashi et al., 2000; Min et al., 2010), processes with optimal μ-values between 0.014 h^{-1} and 0.07 h^{-1} tended towards bell-shaped or complex relationships (Hang et al., 2008; Paulova et al., 2012; Potgieter et al., 2010; Yamawaki et al., 2007; Zhang et al., 2005) and for processes with μ_{opt} > 0.08 h^{-1}, product formation was directly proportional to biomass growth (Curvers et al., 2001b; d'Anjou and Daugulis, 2001; Jungo et al., 2006; Schenk et al., 2008). Interestingly, AOX1-controlled product formation, performed with substrate mixtures of either methanol and glucose (Paulova et al., 2012) or methanol and glycerol (d'Anjou and Daugulis, 2001), showed higher specific growth rates and kinetics similar to GAP-processes with glucose.

Selecting data from Table 3 for two different products (as representatives of product classes), an Fab-fragment and serum albumin, great variability in kinetic relationships was demonstrated (Fig. 2). Production of an Fab-fragment of anti-HIV antibody 2F5 (Maurer et al., 2006) and an Fab-fragment of the human antibody 3H6 (Buchetics et al., 2011) was both controlled by the GAP-promoter, and glucose was used as a substrate during continuous culture. For both recombinant products (only varying in the variable antigen binding site), specific product formation rates were comparable. For these processes, GAP-controlled production of secreted Fab-fragments and biomass growth were associated in a hyperbolic-like relationship. In contrast, secretion of glycosylated antibody (IgG1 with heavy and light chain) by a glycoengineered *P. pastoris* strain under the control of AOX1, in a culture with methanol, was negatively growth-associated (Potgieter et al., 2010). With methanol as a substrate, maximum specific productivity was reached at significantly lower specific growth rates than for GAP-controlled production of Fab fragments. For serum albumin, opposite relationships were observed in cultures with methanol or glucose. AOX1-controlled production with methanol as a substrate showed negative growth-related product formation (Kobayashi et al., 2000) whereas for glucose and the GAP-promoter, positive growth-related product formation was observed (Rebnegger et al., 2014).

2.2.1. Recommendations for best practice in process development

Experimental data on recombinant protein production with *P. pastoris*, as published in the literature so far, reflect the following:

- Different production kinetics was determined in similar categories of recombinant products (*i.e.*, several antibodies or serum albumin, *etc.*). The trend is towards a greater similarity between kinetics for different products built by strains of principally the same design and under the same culture conditions (*i.e.*, promoter and substrate combination) than for recombinant products in the same class (Fig. 2). Therefore, when developing processes for strains which have not yet been characterised, promoter use informs decisions on appropriate substrates and process strategies.
- GAP-controlled production of secreted proteins is growth-associated; it typically increases (linearly, directly proportional) with increasing specific growth rates until close to μ_{max}.
  In direct analogy to kinetics of GAP-controlled production, at a first glance it would seem sensible to design high-performance process strategies at the maximum growth performance close to μ_{max}. However, controlling μ in a decreasing manner as time proceeds over the cultivation process is recommended as this strategy leads to the highest productivities and titres (see Section 3.3 for background rationale).
- Product formation kinetics of AOX1-controlled protein production (in contrast to GAP-controlled) is bell-shaped, which may well be related to saturation of the secretory pathway due to high expression levels (promoter and copy number) or to methanol, a less favourable substrate, being used. Moreover, a multitude of relationships between production and growth were found for AOX1-strains.
  Despite the existing knowledge gap for AOX1-controlled product formation, process design at a low specific growth rate of $<0.04\ h^{-1}$ is particularly suitable for reaching high titres and productivities.

3. Development and implementation of an optimum process strategy

In general, recombinant protein production responds to variations in biomass growth. However, cultivation conditions during the initial screening experiments and those in the production process in bioreactors often differ significantly. Translation of screening results and further process optimisation to match bioreactor conditions are, therefore, difficult and laborious tasks (Hesketh et al., 2013; Hohenblum et al., 2004; Marx et al., 2009; Resina et al., 2009). A crucial aspect, still not adequately covered in the current literature, is the need for screening under 'industrially relevant conditions'; this is rarely incorporated into current screening procedures. The major steps from the construction of a recombinant *P. pastoris* strain to the implementation of a production process, which are illuminated in this review article, are:

- screening of clones (or different constructs) with the aim of reducing their number (Section 3.1);
- further characterisation of 'typical' behaviour observed in first screenings in order to obtain in-depth knowledge of biomass growth and product formation (Section 3.2);
- implementation of a production process in fedbatch mode (Section 3.3).

In this review, we consider medium composition, pH and temperature as already being set at their optima with respect to both strain physiology and possible limits of the equipment. If of interest, these aspects are described in some detail elsewhere (Calik et al., 2015; Cos et al., 2006).

3.1. Screening in small scale

The 'best producer' (clone or strain) is typically identified at a very early stage of process development, and is then propagated throughout the remaining developmental phases of the production process of a particular protein. In general, initial high-throughput screening is applied to correct for significant clonal variability (inherent in current *P. pastoris* transformation protocols) and to reduce the considerable experimental load when performing bioreactor experiments. As screening more than 2–4 clones under production conditions in bioreactors is labour-intensive and, thus, impractical, only a few of the 'best' performing *P. pastoris* transformants are typically selected from the initial large population (up to several hundred) (Weis et al., 2004). However, interpretation and direct transferability of results from batch culture screening, in shake flasks or deep-well plates, to fedbatch bioreactor cultures is essential (Barnard et al., 2010; Hemmerich et al., 2014; Wilming et al., 2014). Moreover, it is notable how published protocols for high-throughput screening still treat this step as being separate from core process development.

3.1.1. Screening in batch mode

Screening for and selecting clones is a critical step in the development of a process for producing a heterologous protein in *P. pastoris*. In recent years, screening of microorganisms in general has benefited from a number of technological advances, allowing expansion, even in very simple laboratory settings, of the number of parallel batch-type experiments that can be carried out using 96-deep-well plates or similar devices (Camattari et al., 2014; Weinhandl et al., 2012; Weis et al., 2004).

For AOX1-controlled production, methanol is typically pulsed repeatedly after complete depletion of the pulsed glucose substrate in order to maintain a residual substrate concentration within predetermined upper and lower concentration limits (Weis et al., 2004). During phases of substrate excess (which occur immediately after pulsing of the substrate), biomass grows with a specific growth rate close to its maximum (μ_{max}). Between subsequent pulses, *i.e.*, after substrate depletion, biomass growth typically declines to $0\ h^{-1}$ and biomass concentration stagnates (Fig. 4).

Unlike pulsed strategies applied in screening, in a bioreactor, the specific growth rate is controlled by the rate of substrate addition at a defined value lower than or equal to its maximum (μ_{max}). In this sense, screening in 96-well plates does not reflect the conditions of (industrial) fedbatch cultivation. The optimum for AOX1-controlled product formation, according to current literature on kinetics (Table 3), is often considerably below μ_{max} (Fig. 4). However, for GAP-controlled product formation, optimum specific growth rate is often near to μ_{max}, which is also reached during screening (Table 3). The consequence may be a serious failure to identify clones that will become true 'top producers' under large-scale cultivation conditions. However, identifying a clone able to express large amounts of the desired product does not merely entail selecting the 'best producer' that is appropriate for the final production/manufacturing scale (Mellitzer et al., 2012). High production is generally detrimental to cell metabolism and cell survival (Holmes et al., 2009; Jafari et al., 2011) and, thus, the 'best' producers from initial screening may fail in bioreactor processes. Therefore, clustering clones according to their performance (best, middle, low) and selecting representatives for further characterisation in fedbatch processes is a way of dealing with the high number of clones and simultaneously not losing those with intrinsic variation before testing them in bioreactors (Mellitzer et al., 2012).

For screening product formation controlled by the AOX1-promoter, the use of the Mut^S phenotype can be advantageous (Cos et al., 2005) and results generated in screenings are more likely to be transferable between batch and fedbatch modes. The maximum specific growth rate, and therefore the growth range on methanol, is smaller for Mut^S than for Mut^+ strains. Examples of screening based on an advantageous

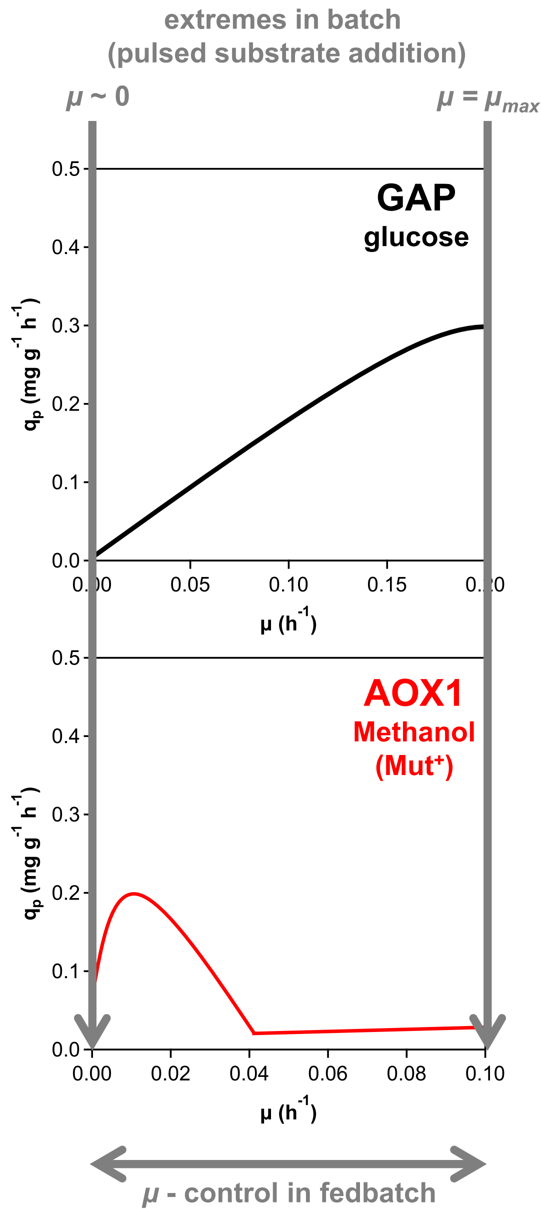

Fig. 4. Screening of clones for recombinant product formation by pulsed substrate addition. Stylised kinetics (according to Table 3) of GAP- and AOX1-controlled product formation are related to typical growth rates achieved during screening with substrate pulsed to an excess concentration (batch) and during substrate-limited mode in the bioreactor (fedbatch).

condition for high producers and on extending the cultivation range for *P. pastoris* are the production of G-protein coupled receptors (Singh et al., 2012b), or intracellular, membrane-associated recombinant products for fungal cytochrome P450 (Gudiminchi et al., 2013).

3.1.2. Overcoming drawbacks of current screening concepts

Production processes based on *P. pastoris* as an expression host are typically more affected by clonal variability than those using either *Escherichia coli* or *Saccharomyces cerevisiae*. There are three main reasons for this specific feature of *P. pastoris* processes. Firstly, it is widely known that clonal differences are amplified in chromosomally-integrated systems compared with episomal expression cassettes; genomic perturbances represent a strong source of background noise (Liachko and Dunham, 2014). Expression systems largely based on episomal vectors, such as in *E. coli* or *S. cerevisiae* (at least during strain selection), are less prone to clonal variability due to genomic perturbance. Secondly, and particularly pertinent to 96-well cultivation, results gained from several small scale expression methods need to be carefully evaluated due to the presence of 'edge effects', *i.e.*, micro-variability at different positions on a 96 well plate, typically affecting clones growing in wells on the outer edge (Lundholt et al., 2003). Such artifacts are observed with a variety of different hosts and, though relevant, are not exclusive to *P. pastoris* cultivations. A third effect impacting on clone behaviour in small-scale cultivations is the fact that methanol induction causes a strong transient response that is unique to each strain. Such a response is, by its very nature, affected by cell-to-cell variability, and is difficult to normalise when considering a population of cells, even of the same genetic clone, and represents phenotypic variability (Hesketh et al., 2013)

A bioreactor offers a much higher degree of process control and monitoring possibilities than 96-well plates and shake flasks. The most important difference between small-scale screening and bioreactor cultivation is the ability to control the specific growth rate at values below its maximum. The gap between screening in batch culture mode (in shake flasks or deep-well plates) and the subsequently performed, labour-intensive, bioreactor culture in fedbatch mode is currently addressed by small-scale bioreactors and new feeding techniques (Barnard et al., 2010; Lattermann and Buchs, 2014; Wilming et al., 2014). Down-scaled techniques of substrate addition include pump-controlled feeding (Barnard et al., 2010), release of glucose from a polymer (Hemmerich et al., 2014) or by an enzyme (Panula-Perala et al., 2008), and microfluidic systems (Grunberger et al., 2014). These fedbatch-like screening small-scale techniques better reflect conditions in the bioreactor to be used later in production scale. However, polymer-based and enzymatic release systems are not available for the controlled release of methanol.

3.1.3. Recommendations for best practice in process development

The quote 'You get what you screen for' (Schmidt-Dannert and Arnold, 1999) best reflects the dilemma of screening, which in principle dictates a 'quantity-over-quality' approach. Nevertheless, the following recommendations may facilitate good decisions based on information obtained from screening:

- Proper screening is crucial and needs to be carried out before cultivation process development. However, culture conditions under high-throughput screening (in shake flasks or deep-well plates) are quite different from fedbatch bioreactor cultures and therefore the 'best' producing clone identified in screening will not necessarily be the best performing one in a bioreactor. Though its use is labour-intensive, a bioreactor offers a much higher degree of process control and monitoring than deep-well plates and shake flasks.
- Typically, the optimum μ-values for product formation with methanol (based on Table 3) are below maximum specific growth rates achieved during pulsed screening in deep-well plates. Thus, in batch cultures, optimum performance of Mut+/AOX1-strains cannot be achieved and clusters of low, middle and high producing clones should be selected for further testing.
- AOX1/Mut^S-strains, in which the ability to grow with methanol is deliberately impaired, exhibit a narrow range of specific growth rates, and therefore the results of screening in batch mode (with methanol pulses) may be transferable to a fedbatch production process.
- The results of screening GAP-strains in batch cultures are typically transferable to fedbatch, since the production optimum is close to μ_{max}.

3.2. Characterisation of biomass growth and product formation

Optimum conditions for the production of a recombinant protein in *P. pastoris* differ according to the target molecule and promoter (Hyka et al., 2010). To develop a production process for a newly constructed strain of unknown behaviour, it is therefore essential to start with its physiological characterisation. Since lacking a theoretical foundation, such a characterisation requires an empirical understanding of the relationship between growth and product formation. Only based on

knowledge of product formation kinetics can the appropriate production conditions (*i.e.*, both optimum range and time course of the specific growth rates) be identified and implemented by controlled substrate addition in a fedbatch process.

3.2.1. Determination of maximum specific growth rate

Maximum specific growth rate (μ_{max} in units of h^{-1}) is a critical parameter for designing an appropriate and specific process strategy, since it determines the maximum substrate consumption and thus, the upper limit of substrate addition in a fedbatch feed profile. However, recombinant strains engineered for production of heterologous proteins typically exhibit maximum specific growth rates (Table 2) that are significantly lower than those observed with a non-engineered strain.

In order to avoid substrate accumulation during fedbatch processes, μ should be controlled at values below μ_{max}, which are strain specific and therefore, only achievable by experimentation. Maximum specific growth rate can be determined directly during batch cultivation (Fig. 1), in which substrate is available in excess and thus biomass growth is unrestricted. For AOX1-controlled product formation, batch experiments with an initial methanol pulse of 0.5% (v/v) and at least 4 consecutive pulses of 1.0% (v/v) methanol were performed to determine both the maximum specific growth rate and the maximum specific substrate uptake rate (Dietzsch et al., 2011b). With the initial methanol pulse of 0.5% (v/v) clone-specific adaptation time to methanol was determined (Dietzsch et al., 2011b; Hesketh et al., 2013). Depending on the pulse-strategy, biomass growth either stagnates between pulses (after substrate depletion, μ approaches 0 h^{-1}) or growth approaching μ_{max} is held throughout by immediate repeated pulses after substrate depletion. In principle, complete substrate depletion is indicated by a sudden increase in dissolved oxygen, and a drop in carbon dioxide concentrations, as monitored by off-gas analyses.

Dietzsch et al. (2011b) conducted two subsequent 1.0% (v/v) methanol pulses (by immediate pulsing after substrate depletion) and repeated this approximately every 24 h. According to a Monod-type relationship, maximum specific growth rates were reported to be achieved at methanol concentrations of between 0.4% (v/v) and 0.5% (v/v) (Curvers et al., 2001b; Kobayashi et al., 2000; Zhou and Zhang, 2002). A negative effect on cell growth was measured for methanol concentrations of >0.4% (v/v) (Zhou and Zhang, 2002), >0.9% (v/v) (Kobayashi et al., 2000), 1.5% (v/v) (Minning et al., 2001) and 3.0% (v/v) (Khatri and Hoffmann, 2006a). However, with methanol concentrations of <0.13% (v/v), cells grew according to the model of Curvers et al. (2001b), with specific growth rates below μ_{max}. Despite this rather inconsistent information on inhibitory concentrations of methanol, repeated pulses of 1.0% (v/v) methanol were found to be an appropriate concentration for determining μ_{max} as well as $q_{s,max}$.

However, production characteristics such as titres, yields and specific productivities obtained for this set of operational parameters used to determine μ_{max} for Mut^+ and Mut^S strains/clones (Dietzsch et al., 2011a; Krainer et al., 2012) do not reflect the highest possible productivity (Table 3). The best producing strain/clone could not therefore, be selected based on q_p-data obtained in such batch experiments.

3.2.2. Establishing production kinetics

Concerning characterisation of AOX1-controlled protein production, bioreactor experiments are required to completely characterise the potential of particular production clones. As a rational basis for process development, the relationship between specific product formation rate and the corresponding specific growth rate has to be determined *de novo* and empirically for each strain/clone.

Generally, numerous laborious and time-consuming fedbatch (Kobayashi et al., 2000; Potgieter et al., 2010) or continuous cultivations (Jungo et al., 2006; Paulova et al., 2012) are performed at several different pre-set μ-values to establish the desired $q_p(\mu)$-relationship (Fig. 2). Fedbatch strategies for continuous addition of an organic carbon and energy source are usually based on mathematical functions describing time dependency on the rate of substrate addition (*i.e.*, the feed profile, Fig. 5). In such strategies, substrate is added at predefined rates that increase or decrease following linear or exponential functions, or by equivalent stepwise approximations (Fig. 5). Unlike pulsed strategies (Dietzsch et al., 2011a), the specific growth rate is controlled by the rate of substrate addition at a defined value lower than or equal to its maximum (μ_{max}) (Zhang et al., 2007). The added substrate is immediately utilised and therefore cells can only grow as fast as the rate of substrate supply. In order to maintain a constant specific growth rate over the entire course of a fedbatch process (Fig. 1), the feed rate must be increased exponentially (the biomass also grows exponentially) providing a constant amount of substrate CDW^{-1} h^{-1} (Potgieter et al., 2009).

Optimum production conditions for AOX1-controlled recombinant product formation with Mut^+ strains are recommended to be determined at constant μ of 0.2 h^{-1}, 0.4 h^{-1}, 0.6 h^{-1} and 0.8 h^{-1}, *i.e.*, each μ studied during a single exponential fedbatch cultivation (Zhang et al., 2007). For Mut^S strains with a considerably lower strain-specific μ_{max} than 0.154 h^{-1} (Kobayashi et al., 2000) these pre-set μ values would be too high. In Table 3 the authors referenced only two $q_p(\mu)$-relationships for which the productivity maximum was at μ of about 0.14 h^{-1} (Jungo et al., 2006; Schenk et al., 2008). Typically production kinetics were investigated for $\mu < 0.08$ h^{-1} (Curvers et al., 2001b; Zhang et al., 2005) or even below 0.03 h^{-1} (Hang et al., 2008; Kobayashi et al., 2000; Min et al., 2010; Potgieter et al., 2010). Possibly the relatively low specific growth rates were chosen since they are favourable for the design of a production process with a high titre and productivity (Mallem et al., 2014). For fully growth-associated GAP-regulated production, kinetics was typically investigated between 0.02 h^{-1} and 0.19 h^{-1} (Buchetics et al., 2011; Maurer et al., 2006).

Establishing production kinetics with fedbatch cultivations at several different pre-set μ-values is laborious and time-consuming, and, therefore, alternative approaches are being sought. The use of dynamic process conditions for fast physiological strain characterisation are summarised by reviews of Spadiut and Herwig (2014) and Spadiut et al. (2013). Various dynamic fedbatch approaches currently under development would allow the entire production range of μ to be covered in a single experiment (Lüthy et al., 2011; Spadiut et al., 2014b; Zalai et al., 2012). In these kinds of experiments, how product formation may adapt with time has also been investigated (Spadiut et al., 2014b). For example, a steep increase in specific product formation rate was observed 40 h after methanol induction, independent of the feeding strategy applied (Spadiut et al., 2014b). Thus for approaches with constant or dynamically adapted μ-values, time after induction in which specific productivity is stable must be carefully determined in order to incorporate time dependency of product formation into the design of the fedbatch production process (Dietzsch et al., 2011a; Meyer and Schmidhalter, 2014; Spadiut et al., 2014b).

3.2.3. Recommendations for best practice in process development

The author's own experience in developing recombinant protein production with *P. pastoris*, and supported by extensive literature research, leads to the following recommendations:

- Physiological strain characterisation is the mandatory first step of any product development. Since such characteristics are strain/clone-specific, it is not predictable *a priori* and each strain should be experimentally characterised *de novo*.
- The strain-specific maximum specific growth (μ_{max}) rate is a critical parameter restricting maximum possible feed addition in order to avoid substrate accumulation. Repeated pulses of 1.0% (v/v) methanol in batch cultures were found to be an appropriate and easy-to-handle approach for determining μ_{max}.

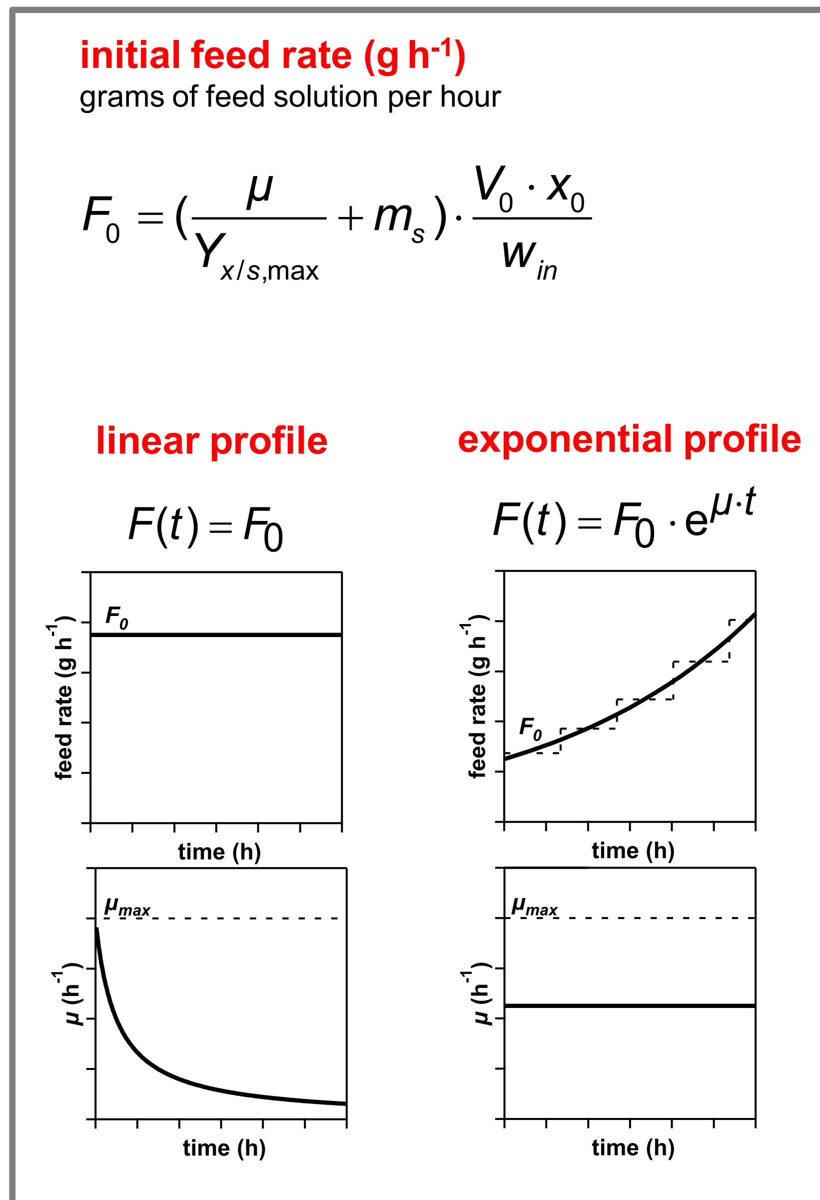

Fig. 5. Calculation box: Feed profile.

- Knowledge of production kinetics, and the $q_p(\mu)$-relationship, are essential to the design of a fedbatch production strategy in which growth is controlled at a certain optimum μ-value < μ_{max} by the controlled addition of carbon-substrate.
- The $q_p(\mu)$-relationship cannot be established in batch cultures. For AOX1-controlled product formation, fedbatch cultivations at pre-set specific growth rates of 30%, 50% and 10% of μ_{max} (*i.e.*, this recommended order is practical and a fourth μ optional), which are achieved by exponential addition of substrate, should be held constant during the experiment. Such a systematic development of three exponential fedbatch processes would allow an early and efficient assessment of product formation kinetics, as well as avoiding unnecessary additional cultivations and variations in the final results. For fully growth-associated, GAP-controlled production, fedbatch experiments at relative specific growth rates of 25%, 50% and 75% of μ_{max} are recommended.
- For a long-term perspective, applications of dynamic fedbatch feeding strategies are very promising and time-saving, but more experience in establishing and producing reproducible results is required than for exponential fedbatch cultivations.

3.3. Establishing fedbatch production processes

Historically, the strong and tightly regulated AOX1-promoter (*pAOX1*) has been mainly used for recombinant protein expression in *P. pastoris* (Ahmad et al., 2014; Macauley-Patrick et al., 2005). Using AOX1, methanol acts as an inducer for recombinant protein production and, at the same time, as a carbon and energy source. Thus, induction and production of heterologous protein are interconnected with substrate utilisation and biomass growth. Moreover, purpose-engineered AOX1-phenotypes that relate to the efficiency of methanol utilisation (*i.e.*, Mut$^+$ and Mut^S strains, Section 2) represent an additional opportunity in strain/process design (Cos et al., 2005; Pla et al., 2006). In this respect, the *P. pastoris* expression system can be differentiated from, for example, *E. coli*, in which promoters can be induced by an independent, non-metabolisable agent. Consequently, *P. pastoris* production processes are more complex to control.

Commercially available expression kits by Invitrogen were crucial for the development of the *P. pastoris* expression system and still dominate the standard protocols for AOX1-controlled (*i.e.*, inducible) production of heterologous proteins (Invitrogen, 2002; Stratton et al.,

1998; Tolner et al., 2006). The typical *P. pastoris* cultivation process, therefore, follows a three-stage strategy (Fig. 5):

- a batch phase for biomass growth with glycerol or glucose,
- a fedbatch phase for further biomass enhancement with glycerol or glucose and
- an optional methanol-induced adaptation (transition) phase, which is followed by a production phase in fedbatch mode.

3.3.1. Standard protocols for AOX1-controlled product formation

Conventional *P. pastoris* protocols describe feeding profiles for 'simple' recombinant protein production without customised design of the feed profile (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006). What standard protocol is suitable for a particular strain depends on the strain-specific maximum specific growth rate with methanol and the optimum μ-operational range for product formation.

From the three fedbatch protocols often used (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006), time courses of biomass production, and from these, specific growth rates were calculated and are shown in Fig. 6. Time courses of biomass (in cell dry weight, CDW) were calculated using biomass to substrate yields of 0.61 g g^{-1} for glycerol (Jungo et al., 2006) and 0.33 g g^{-1} for methanol (Zhang et al., 2005). A maintenance rate for methanol of 0.016 g g^{-1} h^{-1} from a recombinant strain with μ_{max} of 0.07 h^{-1} was used (Zhang et al., 2005). These values were assumed to be typical but may, in fact, vary for each new strain (Table 2). For all three protocols, the rate of methanol addition was increased stepwise during the first 10 h of production and then kept constant (Fig. 6). When the feed rate is kept constant, the amount of substrate CDW^{-1} h^{-1} actually decreases during the process, resulting in a decreasing specific growth rate with time (Fig. 5, Fig. 6). One of the main differences between the protocol of Tolner et al. (2006), that was described in the Invitrogen protocol (2002), and the *P. pastoris* protocol book by Stratton et al. (1998), is the lower specific growth rate throughout the entire process following the initial stepwise increase over approximately 6 h. Maximum values of μ of about 0.04 h^{-1} (Stratton et al., 1998), 0.05 h^{-1} (Invitrogen, 2002), and 0.02 h^{-1} (Tolner et al., 2006) were theoretically reached at the beginning of constant methanol addition and then decreased to values of $<$0.01 h^{-1} (Fig. 6). Profiles for Mut^S promoted lower initial specific growth rates of around 0.02 h^{-1} (Stratton et al., 1998), or 0.01 h^{-1} (Invitrogen, 2002) and then decreased to values of $<$0.01 h^{-1}. Time

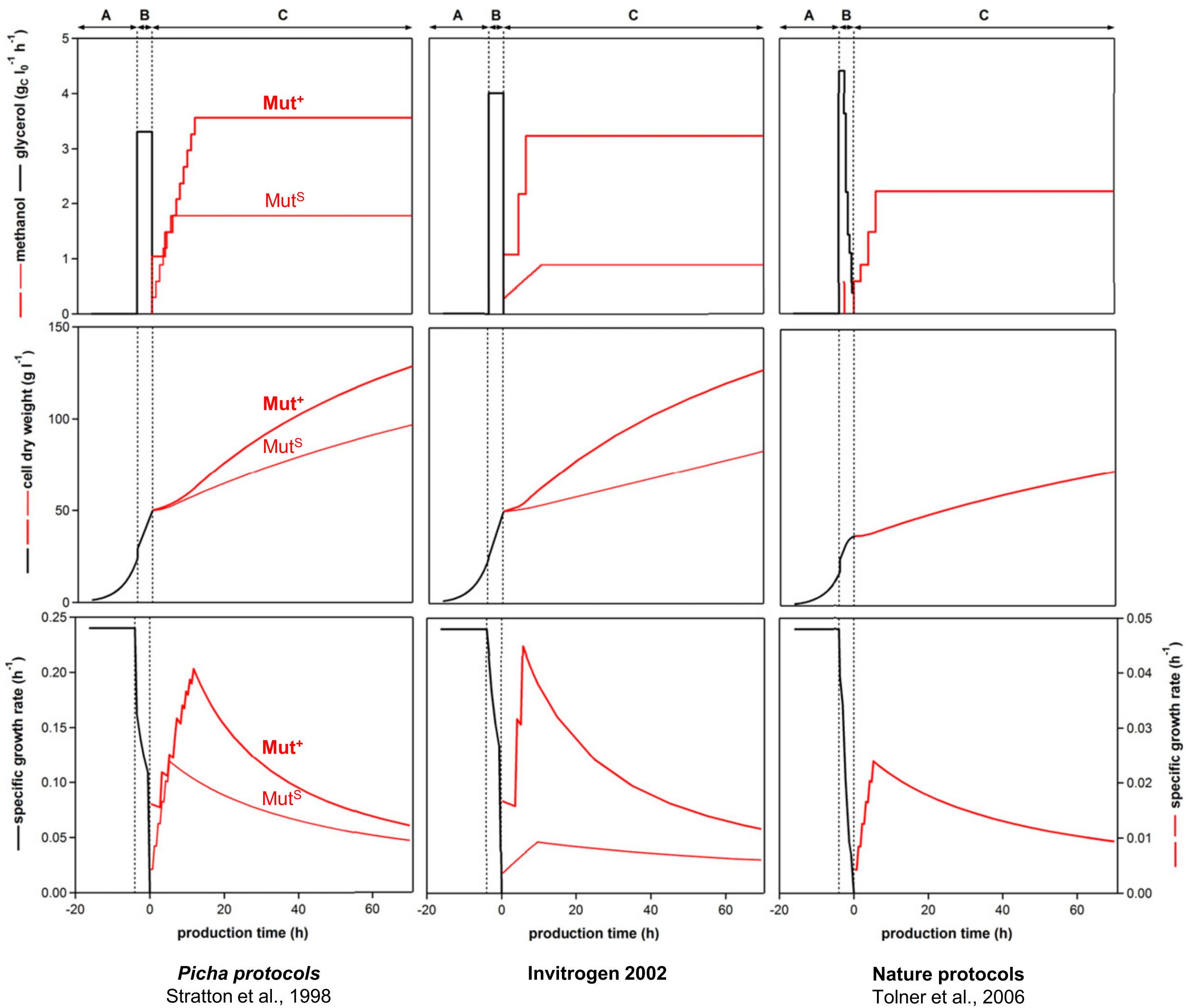

Fig. 6. Operational ranges of standard protocols for AOX1-controlled product formation (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006).First row: 3-stage culture with a batch phase for biomass growth with glycerol (A), a fedbatch phase for further biomass enhancement with glycerol and AOX1 derepression (B), and a production phase in fedbatch mode using methanol (C) are described. In the protocols, time courses are given in grams of carbon (of the corresponding substrate) per initial volume in the process phase, per hour.Second row: time courses of dry cell dry weight were calculated using biomass to substrate yields of 0.61 g g− 1 for glycerol (Jungo et al., 2006) and 0.33 g g− 1 for methanol (Zhang et al., 2005). A cell maintenance rate for methanol of 0.016 g g− 1 h− 1 was used (Zhang et al., 2005). An initial glycerol concentration was set to 40 g l− 1 in batch, and the fedbatch phase (B) was finished when 50 g l− 1 biomass was reached.Third row: left axis corresponds to specific growth rates on glycerol (black lines) and right axis to specific growth rates on methanol (red lines). These theoretical time courses of specific growth rates were calculated from the time courses of biomass concentrations (second row).

courses of specific growth rates can differ significantly if initial biomass concentrations in the production phase are changed due to variations in protocols. For this calculation, an initial CDW concentration of 50 g l^{-1} was assumed according to Invitrogen protocols (2002) (180–220 g l^{-1} wet cell weight (WCW)). However, the initial feed rate, F_0, can be adjusted to any biomass concentration according to Fig. 5.

3.3.2. Implementing customised process strategies

In recent years, protocol design has shifted from classical 'recipes' (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006) to more conceptual attempts (Maurer et al., 2006; Zalai et al., 2012; Zhang et al., 2007). Considering the maximum specific growth rate (μ_{max}) as the upper limit and the production kinetics $q_p(\mu)$ as an indicator of the optimum production range of μ, design of a customised fedbatch feed profile to maximise product titre is possible (Fig. 7).

With *P. pastoris* as the expression host, several gram per litre of recombinant enzymes have been produced during fedbatch production processes (Table 4). The highest published titres for Fab-fragments and hormones were in the range of 1 g l^{-1} to 4 g l^{-1} (Table 4). However, high product titres are partly due to high biomass concentrations and long production times (Table 4). At the beginning of the methanol induction-/production-phase, CDW concentrations between 20 g l^{-1} to 100 g l^{-1} were reported (Table 4). The biomass concentration at the beginning of the production phase is a critical factor that should be considered for optimising high-level production (Mallem et al., 2014; Zhang et al., 2007). By Invitrogen protocols (2002), an initial biomass concentration of 180 g l^{-1} to 220 g l^{-1} WCW, which corresponds to approximately 50 g l^{-1} CDW, is recommended. However, in the second edition of *P. pastoris* protocols 2007, Zhang et al. (2007) recommend higher initial biomass concentrations between 150 g l^{-1} to 450 g l^{-1} WCW, corresponding to 38 g l^{-1} to 113 g l^{-1} CDW.

Up to what biomass concentrations is the recombinant protein formation physiologically and practically feasible has still to be elucidated. Furthermore, biomass separation from high cell density cultures is a challenging task in downstream processing (Meyer and Schmidhalter, 2014). The highest final biomass concentration reported for *P. pastoris* grown on methanol, continuously added in fedbatch culture, was 150 g l^{-1} (Curvers et al., 2001a). In fedbatch with glucose, a biomass concentration of more than 200 g CDW l^{-1} was achieved (Heyland et al., 2010).

Generally, high productivity and a high final titre (Table 4) are reached if cultivation occurs at a high biomass concentration, for long periods, at a desired μ for product formation, before system boundaries are reached (*i.e.*, maximum biomass concentration, maximum heat evolution or oxygen supply) (Fig. 7). However, this is only the case if high productivity is reached at low specific growth rates. AOX1-controlled product formation typically shows higher specific productivities at lower specific growth rates. Maximum specific productivities within the range of 0.1 mg g^{-1} h^{-1} to 0.26 mg g^{-1} h^{-1} were reported at specific growth rates below 0.02 h^{-1} (Bhatacharya et al., 2007; Hang et al., 2008; Kobayashi et al., 2000; Potgieter et al., 2010; Yamawaki et al., 2007). GAP-controlled processes tend to exhibit high specific product formation rates at a level comparable to AOX1-strains but for an operational μ near μ_{max} (Fig. 2, Table 3). Along with this consideration Buchetics et al. (2011) reverse engineered a strain with increased GAP-controlled high product formation at low specific growth rates (<0.1 h^{-1}).

Established product formation kinetics has been the basis for several attempts at rational process design and optimisation (Kobayashi et al., 2000; Maurer et al., 2006; Zhang et al., 2005). For instance, productivity (r_p) at the end of the process was maximised using a solver-based MS-Excel file application, by finding the best time course for a specific growth rate and duration of the process (Maurer et al., 2006). However, the application was for GAP-controlled recombinant protein production with a single substrate for growth and production. For AOX1-controlled processes, biomass concentration at the start of the methanol induction-/production-phase is variable and has to be optimised, together with the time course for μ during the production phase (Mallem et al., 2014; Zhang et al., 2007). A maximisation model for AOX1-controlled processes was established by Kobayashi et al. (2000) and Zhang et al. (2005). Optimal time courses to reach maximum product formation, in all three attempts, showed the following typical profile: high specific growth rate at the beginning of the process to increase biomass concentration and a subsequent decrease during the production phase.

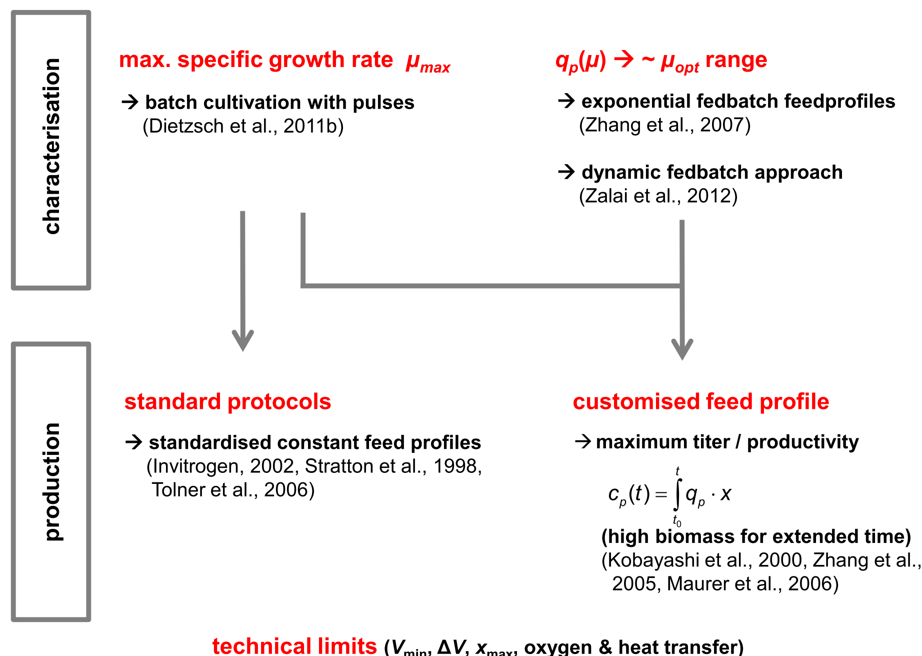

Fig. 7. Concept for a rational cultivation strategy based on product formation kinetics.

Table 4
Operational settings for high productivity fedbatch production processes.

$c_{p,end}$ ($g\,l^{-1}$)	Heterologous product	Time[a] (h)	$CDW_{induction}$[b] ($g\,l^{-1}$)	CDW_{end} ($g\,l^{-1}$)	μ (h^{-1})	Operation mode	Substrate	Promotor	Strain	References
Enzymes										
19.55	Glucose oxidase (*Aspergillus niger*)	168	100	245	NA	Feedback control (MeOH conc. at 1.8 v/v%)	Methanol/ mannitol	AOX	GS115	Gu et al. (2015)
5.8	Lipase/acetyltransferase (*Escherichia coli*)	119	20	74	0.01	Feed forward (exponential)	Methanol	AOX	GS115	Brunel et al. (2004)
3.3	Lipase (*Yarrowia lipolitica*)	77	–	120	NA	Feedback control (DO level between 25–35%)	Glucose	GAP	X-33	Wang et al. (2012)
~2.5	Cellobiohydrolase 2 (*Trichoderma reesei*)	90	–	NA	NA	Feed forward (linear)	Glucose	GAP	CBS7435	Mellitzer et al. (2012)
Antibody										
2.116	Fab fragment (human antibody 3H6)	~25	–	100	0.15–0.05	Feed forward (exponential and linear)	Glucose	GAP	X-33	Buchetics et al. (2011)
1.7	IgG1	182	~30[c]	~138[c]	0.014	Feed forward (exponential)	Methanol	AOX	YGLY4140	Potgieter et al. (2010)
0.198	scFv fragment (anti-bisphenol A)	~30	~60	~80	NA	Feedback control (MeOH conc. at 0.5 v/v %)	Methanol	AOX	GS115	Yamawaki et al. (2007)
0.046	Fab fragment (anti-HIV antibody 2F5)	92	–	96	0.2–0.05	Feed forward (exponential and linear)	Glucose	GAP	X-33	Maurer et al. (2006)
Hormone & growth factors										
3.84	Insulin precursor	~130	56	59	NA	Feedback control (MeOH conc. at 0.25 v/v %)	Methanol	AOX	X-33	Gurramkonda et al. (2010)
0.97	Insulin precursor (porcine)	80	59	~140	0.016	Feed forward (exponential)	Methanol	AOX	GS115	Hang et al. (2008)
0.76	GM-CSF (murine)	68	~25[c]	~55[c]	0.015	Feedback control (exponential)	Methanol	AOX	GlycoSwitch-Man5	Jacobs et al. (2010)
Interferone & interleukin										
0.25	rhIL-2-HSA fusion	60	–	250 (OD_{600})	NA	Feedback control (DO level between 25–40%)	Glucose	GAP	GS115	Guan et al. (2013)
0.392	Interferone (ovine)	70	NA	~117[c]	0.025–0.020	Feed forward (exponential)	Methanol	AOX	X-33	Sinha et al. (2003)

NA not available.
~Values approximated from figures.
[a] Time of product formation during fedbatch cultivation.
[b] Not stated for constitutive GAP-controlled product formation.
[c] Published parameter adapted from wet cell weight to dry cell weight by a factor of four.

Oxygen and/or heat transfer are most often the limiting factors in high cell density *P. pastoris* cultivations (Cunha et al., 2004), irrespective of the substrate used. However, heat evolution and oxygen uptake strongly depends on specific growth rates and, therefore, the process must be designed within the technical limitations of the available equipment (Fig. 7).

3.3.3. Recommendations for best practice in process development

Specific productivity (q_p), maximum biomass in the reactor ($x \cdot V$), and productive time (*i.e.*, between induction and harvest) are critical factors influencing the performance of biotechnological production processes that aim at producing a maximum amount of product, within desired quality specifications, and over a minimum time. Our collected experience leads to the following recommendations:

- Process strategies described in the available standard protocols cover nearly all μ-ranges typically found for optimal AOX1-controlled product formation (Table 3). Standard Mut^{+} strategies (Invitrogen, 2002; Stratton et al., 1998; Tolner et al., 2006) are suitable for strains with a μ_{max} higher than 0.05 h^{-1}. The Mut^{S} strategies (Invitrogen, 2002; Stratton et al., 1998) are suitable for strains with a maximum specific growth rate higher than 0.02 h^{-1}.
- Generally, a high initial concentration of biomass and a low specific growth rate during production are favourable. Suitable strategies for maximum product formation and titre show a typical pattern: high initial specific growth rate to rapidly grow biomass to a high concentration, and a subsequent decrease in μ during production, preferably at the optimum μ-value for maximum productivity.
- The current trend is to move away from standard protocols towards concepts that enable an operator to adapt a particular recommendation to any specific clone/strain or bioreactor. This review attempts to address this issue.

4. Future development

In spite of ever increasing interest in and current intensive research on the *P. pastoris* system, it is still far from achieving the maturity of, for example, *E. coli* or *S. cerevisiae*. In particular, from the process development perspective, which is the focus of this review, attempts to reduce the typically high workload required to determine the $q_p(\mu)$-relationship as well as the further development of methods to enhance the robustness of process control on a manufacturing scale are necessary.

In this chapter just a glimpse of a couple of innovative developments are depicted: characterisation of product formation at the single-cell level, and use of software-sensors to reduce workload and enhance product quality (in particular of complex proteins) by control of growth/process conditions. However, they are treated briefly, since each of these topics could be extensively reviewed on its own.

4.1. Single cell level perspective on production

Recent evidence has shown how epigenetic factors can influence the distribution of protein production within a single population (Love et al., 2012). A new paradigm for screening therefore needs to take into account such information, considering the 'best producer' at the

single cell level, since this is the level at which clonal differentiation takes place. The most appropriate method of choice to screen for the 'best producer' in a population, fluorescence activated cell sorting (FACS), enables the identification of satisfactory clones for several processes (Mattanovich and Borth, 2006; Sleiman et al., 2008). However, sorting is affected by a major limitation: secreted proteins, the most industrially appealing products, by their very nature leave the producing cells, and a method based on intracellular fluorescence might fail to select the best producer for secreted products. For intracellular or surface proteins, FACS retains all its validity and potential. Moreover, FACS provides a picture-frame view of production capability in a single cell at a given time; secretion, however, is a dynamic process, adapting to cell metabolism and growth, best captured in single cells (and their progeny) over time (Love et al., 2010). A compromise between the uniformity and speed of analysis by FACS and traditional cultivation (with its loss of information at the single cell level) is provided by the technique of micro-engraving, an emerging method for high-throughput analysis of secreted products from single cells (Love et al., 2006). Micro-engraving is a soft lithographic method based on intaglio printing to generate microarrays comprising secreted products from single cells, and has been used for screening a variety of secreted recombinant proteins (Panagiotou et al., 2011) as well as to characterise the secretome of B cells (Story et al., 2008). Interestingly, this method provides useful insights into secretion dynamics, showing, for example, how such a process is not linked to the cell cycle, but varies stochastically within a single cell during cell duplication.

4.2. Approaches to reduce experimental load

To support laborious and time-consuming physiological characterisation of cells based on sophisticated process control, software-sensors have been established (Brühlmann et al., 2015; Herwig et al., 2001; Jenzsch et al., 2006; Luttmann et al., 2012). Such software-sensors deliver information on the important real-time variables that characterise a bioprocess (such as concentrations of substrates, products and biomass) and are typically determined off-line, with an expected post-sampling delay of several hours. Available on-line data led to advanced process controls based on real-time physiological values (*i.e.*, specific rates of biomass growth, substrate utilization, product formation *etc.*), and, therefore, to improved bioprocess reproducibility and higher product quality. Besides a reduction in the experimental load, human handling errors (*e.g.*, from pipetting or other sampling procedures) are reduced, and higher sampling frequency is achieved, which results in enhanced bioprocess-data quality. In particular, the possible 'automated' determination of specific maintenance rates (m_s) would be an asset for the rational design of appropriate process strategies.

4.3. Product quality

The term 'product quality' takes into account target attributes (both desired and unwanted), which may belong to the following categories (Eon-Duval et al., 2012):

- biological activity, half-life, immunogenicity and safety;
- deamination, glycation, glycosylation, oxidation, hydroxylation and the formation of disulphide bonds, aggregation, *etc.*

Therefore a correctly folded and functional (active) protein, whose post-translational modifications are both correct and sufficient, is the product (Fig. 8) that should be finally quantified to reveal true production kinetics. For example, a correctly glycosylated protein should have the correct human-like mannose-5 structure (*i.e.*, as already engineered for the *P. pastoris* system; Jacobs et al., 2009; Vervecken et al., 2004) and all glyco-sites should be occupied (Ha et al., 2011b). In addition, possible degradation of the protein, both intra- and extra-cellularly, should be considered when establishing kinetic relationships (Celik et al., 2009). Although such sophisticated analyses are demanding and not always available, an alternative, acceptable, mathematical description of product formation kinetics is still lacking. A few examples of recent experimental studies show and quantify effects of culture conditions such as specific growth rate, substrate composition, temperature and oxygen supply on correct and sufficient post-translational protein modifications (Ha et al., 2011a; Hesketh et al., 2013; Jacobs et al., 2010; Rebnegger et al., 2014; Schenk et al., 2008; Seman et al., 2014; Wu et al., 2011, 2012).

Examples of complex proteins of high scientific and commercial relevance are human membrane proteins. Although the strength of the *P. pastoris* system lies in the production of secreted proteins (as described in the previous chapters), several applications as an efficient host for intracellular production of heterologous mammalian membrane proteins (MPs) have been reported in the literature (Bornert et al., 2012; Byrne, 2015; Hedfalk, 2013; Singh et al., 2012a). Human MPs are highly desirable for pharmaceutical research because of their importance in human pathophysiology, but have not been readily available in the required quantities (*i.e.*, milligram amounts) or crystallisation-grade quality until recently, following developments in advanced recombinant technologies (Krettler et al., 2013).

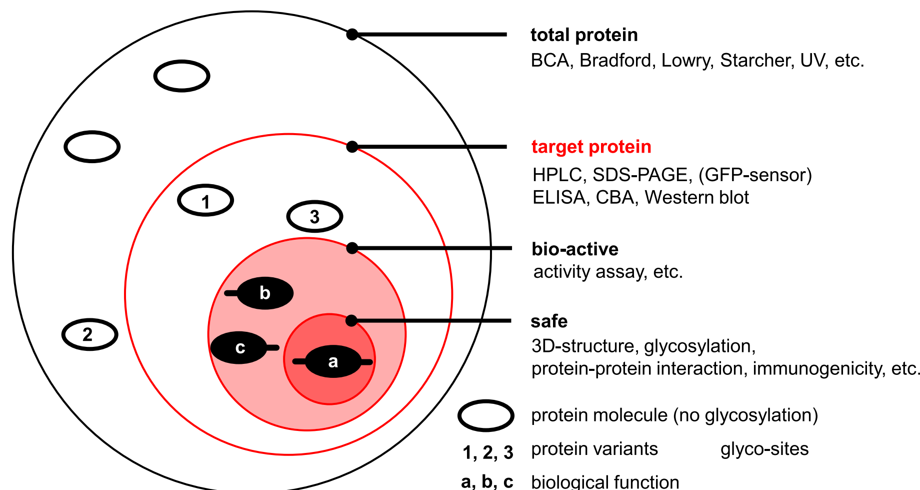

Fig. 8. Schematic presentation of different (molecular) levels of quantitative protein analyses. (*e.g.*, from a rather non-specific total protein quantification to quantification of proteins with correct glycoforms).

A prominent example of the successful production of a stable and functional MP in *P. pastoris*, which allowed its crystallisation and structural determinations, is the human heteromeric amino acid transporter 4F2hc/LAT2 (Costa et al., 2013; Meury et al., 2014). Heteromeric amino acid transporters (HATs) are common to all kingdoms of life, and are composed of two different subunits (heavy and light chains, 4F2hc and LAT2 respectively) linked by a conserved disulphide bridge (Fotiadis et al., 2013). The heterodimer 4F2hc/LAT2 was successfully overexpressed in *P. pastoris*, strain KM71H, including the required post-transcriptional modifications by co-transformation of both subunits (Costa et al., 2013; Rosell et al., 2014). However, the correct (and stoichiometric) linkage of the subunits by the disulphide bridge was affected by culture conditions, as studied systematically in bioreactor cultures (unpublished results). Finally, MPs are one of the emerging applications of the *P. pastoris* system, where use of the concepts outlined in this review may become relevant.

5. Conclusions

The aim of this review is to establish the main principles for both comparison and development of biotechnological production processes with *P. pastoris*. The conclusions, which follow a trend away from standardised recipes to a concept transferable between both different strains and bioreactor systems, are drawn from multiple perspectives, and in particular highlight cell physiology, product classes, promoters, and the process strategies applied. To achieve this, a multitude of published data was analysed, evaluated (recalculated) and compared.

Moreover, the 'recommendation' paragraphs closing each subchapter are of practical relevance by guiding the reader through best practices for bioprocess design, which comprise the aspects of strain/clone screening, strain physiological characterisation and implementation of a fedbatch process strategy. Particularly highlighted is the rationale for why results from screening in micro-titre plates are only rarely directly transferable to production processes in bioreactors.

Our aim is to foster a greater understanding of scalable, and thus transferable process characteristics. The relationship between biomass growth and protein production, which results from both genetic strain construction and culture conditions, is the basis for a rational design of an optimum process strategy specific to a combination of a particular product and genetic construct. The observed time course concentrations of secreted proteins typically resulted from balanced flows through transcription, translation, folding and degradation. They were used, together with time courses of biomass concentrations, as a basis for determining production kinetics. This review uniquely compares processes/strains on the basis of production kinetics (*i.e.*, the relationship between specific growth rate, μ, and specific productivity, q_p) instead of commonly used titers, *i.e.*, final product concentrations, which do not reveal information on biomass concentration, specific productivity or process duration; they are therefore not directly comparable between different processes, strains or bioreactor systems. A deeper understanding of the underlying concepts of process comparison and development has become increasingly relevant as new genetic constructs are made available.

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