Challenges and progress towards industrial recombinant protein production in yeasts: A review

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Abstract

Recombinant proteins (RP) are widely used as biopharmaceuticals, industrial enzymes, or sustainable food source. Yeasts, with their ability to produce complex proteins through a broad variety of cheap carbon sources, have emerged as promising eukaryotic production hosts. As such, the prevalence of yeasts as favourable production organisms in commercial RP production is expected to increase. Yet, with the selection of a robust production host on the one hand, successful scale-up is dependent on a thorough understanding of the challenging environment and limitations of large-scale bioreactors on the other hand. In the present work, several prominent yeast species, including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* are reviewed for their current state and performance in commercial RP production. Thereafter, the impact of principal process control parameters, including dissolved oxygen, pH, substrate concentration, and temperature, on large-scale RP production are discussed. Finally, technical challenges of process scale-up are identified. To that end, process intensification strategies to enhance industrial feasibility are summarized, specifically highlighting fermentation strategies to ensure sufficient cooling capacity, overcome oxygen limitation, and increase protein quality and productivity. As such, this review aims to contribute to the pursuit of sustainable yeast-based RP production.

Keywords:

recombinant protein, industrial enzyme, biopharmaceutical, protein transition, yeast, scale-up, process intensification, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*

1. Introduction

Recombinant protein (RP) production is a fast-growing field. In 2020, the industrial enzyme market was valued at 5.96 billion USD and is expected to grow at a CAGR of 7.66% (Verfied Market Research, 2021) while the entire RP market was valued at over 125 billion USD with an expected CAGR of 11.2% (Mordor Intelligence, 2021). This can be attributed to the maturation of several factors within the field of RP production such as the increased number of available production hosts, the availability of advanced genetic manipulation tools, the possibility to perform high-throughput screening of newly developed strains and the growing amount of process optimization studies which have led to an overall increase in process efficiency and industrial feasibility (Madhavan et al., 2021). Furthermore, the so-called protein transition has incentivized the search for alternative, more sustainable ways of protein production and could result in up to 20% of the emission mitigations required to stay below a temperature increase of 1.5 °C by 2050 (Roe et al., 2019; Stehfest et al., 2019). Besides the well-known plant-based protein alternatives, the importance of microbial protein has been steadily growing as well. Indeed, the alternative protein market was valued at 49.7 million USD and is expected to grow at an impressive CAGR of 16.9% to reach more than 126 million USD by 2028 (Vantage Market Research, 2022). This is further demonstrated by the multitude of recent microbial-based protein start-ups such as Those Vegan Cowboys, Nature's Fynd and Perfect Day.

Today, there is a microbial pathway available for the conversion of almost any carbon source into almost any desired target product, and significant progress has been made regarding the expression of a large variety of RP. Yet, these proofs-of-concept often remain within the laboratory environment. To successfully translate these findings to an industrial level, research toward scale-up is crucial. Indeed, scaling a process involves multiple technical challenges such as achieving a sufficient oxygen supply and cooling capacity and dealing with heterogeneous environments, while important capital investments with a high risk level are required (de Lorenzo and Couto, 2019). An increased understanding of these challenges that are inherent to large-scale biotechnological processes is a key aspect for efficient scale-up and to lower production prices to obtain market competitive products. There has been increasing interest in yeasts as versatile production hosts for a wide range of RP such as industrial enzymes, biopharmaceuticals and microbial proteins for food and feed industries (Anchel, 2016; Mattanovich et al., 2012). Therefore, this review aims to provide a comprehensive overview of the state of the art on yeast-based RP production, the remaining challenges and hurdles that limit widespread industrialization, and innovative process strategies to meet these challenges.

2. Yeast production hosts

Yeasts are an interesting and versatile class of RP production hosts. Their eukaryotic nature, which allows these organisms to perform post-translational modifications, makes them applicable for the production of a wide range of RP, a significant advantage over bacteria such as *Escherichia coli* (Mattanovich et al., 2012; Patra et al., 2021; Waegeman and Soetaert, 2011). In addition, as compared to higher eukaryotes such as mammalian or insect cell lines, they are significantly easier to genetically modify, show higher growth rates, obtain higher cell densities, have a lower substrate cost, and are more robust to large-scale fluctuations (Gomes et al., 2018; Potvin et al., 2012). Besides, yeast species are generally known for their higher RP secretion efficiency as compared to bacteria. Extracellular production can be more advantageous as compared to intracellular production hosts in terms of the downstream purification process, an important cost driver that can amount to up to 80 % of the overall RP production process (Labrou, 2014). Indeed, eliminating a cell disruption step avoids the release of intracellular components in the medium facilitating the downstream processing. Additionally, yeasts are a dominating group regarding the production of microbial proteins, such as single cell protein, and have seen wide acceptance on the market, as recently reviewed by Ritala et al. (2017). Within this section, the most prominent yeast species and their relevant characteristics regarding industrial RP production are discussed. This will be confined to *Saccharomyces cerevisiae*, *Pichia pastoris (*or *Komagataella phaffii)*, *Yarrowia lipolytica*, and two *Kluyveromyces* species, i.e., *K. lactis* and *K. marxianus* (Table 1). All have been given the "generally recognized as safe" (GRAS) status by the US Food and Drug Agency (FDA).

2.1. Saccharomyces cerevisiae

Owing to its robustness for large-scale production, *S. cerevisiae* has been widely used for the production of bioethanol, platform chemicals and single-cell protein as well as in bakery, brewing and wine applications and to a lesser extent in RP production (Deparis et al., 2017). Because of its robustness and ease of genetic manipulation, this well-researched model yeast was originally developed as an alternative host for the production of RPs which could not be produced by bacterial hosts (Baghban et al., 2019; Huang et al., 2010). Expression of these RP is typically accomplished using either strong constitutive promoters such as P_{TEF1} and P_{GADPH} or inducible promoters like the galactose-inducible P_{GAL1} (Partow et al., 2010). For a more comprehensive review on the available genetic tools and secretion factors, the reader is referred to Gomes et al. (2018). Nonetheless, *S. cerevisiae* is less attractive for the production of complex proteins due to its characteristic hyper-glycosylation patterns. This can lead to protein heterogeneity, reducing the therapeutic applicability of these proteins and complicating their downstream purification (Jacobs et al., 2009). In addition, challenging protein folding of complex proteins can lead to insufficient protein secretion causing partially degraded products, hereby lowering product yield and further complicating the purification process. To that end, a large amount of research efforts has been dedicated to the improvement of protein secretion of *S. cerevisiae*, and to extension yeasts in general, and has recently been reviewed by Madhavan et al. (2021). Besides, fermentations using this Crabtree positive yeast can lead to undesired carbon losses due to the production of ethanol, thereby lowering the overall process efficiency. These limitations have motivated the investigation of alternative production hosts. As a result, since 2000, a trend reversal in the number of published research papers has been observed with respect to the use of the methylotrophic yeast *P. pastoris* as the expression host for RP production as compared to *S. cerevisiae* (Figure 1). Furthermore, specifically for newly approved pharmaceuticals and from 2018 to 2022, *P. pastoris* was used more frequently as the expression host as compared to *S. cerevisiae*. (Walsh and Walsh, 2022). Yet, owing to its well-known genome and beneficial characteristics listed above, several classes of RP are currently still produced on a commercial scale using *S. cerevisiae*. Examples include

insulin, one of the first and most well-known RP, a wide range of hormones, vaccines and fusion proteins as well as industrial enzymes (G. Wang et al., 2017).

2.2. Pichia pastoris

In the past two decades, *P. pastoris*, also known as *Komagataella phaffii*, has seen a surge in strain development (Pan et al., 2022) and process intensification efforts to boost RP expression (Looser et al., 2014; Yang and Zhang, 2018). Moreover, recent advances in glycoengineering, a tool to improve protein properties through the addition or removal of glycans, have enabled the yeast-based production of complex humanized RP, as extensively reviewed by Ma et al. (2020). The latter include monoclonal antibodies, that are currently mostly produced using mammalian cell lines (Jacobs et al., 2009; Walsh and Walsh, 2022). For *P. pastoris*, many successful glycosylation efforts have been performed in the past couple of decades, turning this methylotrophic yeast into one of the most commonly used yeast hosts for protein expression (Shenoy et al., 2021). Yet, *P. pastoris'* most commonly used promoter system enabling the expression of foreign proteins, i.e., the highly efficient alcohol oxidase 1 promoter (P_{AOX1}), requires methanol as an inducer. The use of methanol has considerable drawbacks, of which the risk of storing and handling large amounts of methanol, as well as the increased heat production and oxygen demands are the most prominent. To address this issue, novel *P. pastoris* strains have been developed. For instance, Mut^S strains, where the *AOX1* gene was knocked out, exhibit a lower methanol consumption rate. As a result, these strains are more desired as compared to Mut^+ wild-type strains. A study comparing both phenotypes for the production of two recombinant enzymes found that the Mut^S strain showed a three-fold higher volumetric productivity and was seven times more efficient in converting methanol into the product (Krainer et al., 2012). Alternatively, methanol-free systems have been developed of which the constitutive glyceraldehyde-3-phosphate dehydrogenase (P_{GAP}) system has been gaining the most attention (Cankorur-Cetinkaya et al., 2018; Vogl and Glieder, 2013). Moreover, novel promoters, such as the innovative methanol-free P_{PDF} and P_{UFF}, are continuously being developed and investigated (Garrigós-Martínez et al., 2021). Several studies have already improved RP production using these alternative promoters as compared to the methanol-inducible P_{AOX1} (Wang et al., 2012a; Zhu et al., 2014). Nevertheless, while constitutive expression can omit the handling of potentially hazardous inducers, toxic proteins are still best expressed by a strong inducible promoter because of the decoupled growth and production phase hence avoiding limitations in end titer and productivity due to product inhibition. For a more comprehensive understanding of promoter engineering for *P. pastoris*, the reader is referred to reviews by Fisher and Glieder (2019) and Ahmad et al. (2014).

The development of humanized RP combined with the availability of strong promoters and efficient cultivation on simple and inexpensive media, has positioned *P. pastoris* as a powerful industrial workhorse. Indeed, *P. pastoris* has a lower maintenance coefficient (m_S) (0.013 g g^{-1} h^{-1}) as compared to *E. coli* (0.04 g g^{-1} h^{-1}), a conventional bacterial RP production host, or *S. cerevisiae* (0.16 g g^{-1} h^{-1}) (Jahic et al., 2002; Rebnegger et al., 2016; Uribelarrea et al., 1990). While, as compared to *S. cerevisiae*, *P. pastoris* may be a more optimal choice for RP production, this yeast has not been studied as extensively and may therefore result in a more extensive process development. For example, additional research focusing on enhancing protein folding and secretion efficiency is required to further increase RP productivity (Yu et al., 2017). In 2009, the first biopharmaceutical RP produced by *P. pastoris* was approved (Çelik and Çalik, 2012; Corchero et al., 2013). Since then, regulatory agencies such as the FDA have approved the production by companies including Sanofi and Merck of over 70 industrial and biopharmaceutical RPs by *P. pastoris* (Lee et al., 2021). A recent overview of industrial enzymes produced by *P. pastoris* in bioreactors up to 50 L scale and RP products on the market is provided by Duman-Özdamar and Binay (2021) and Baghban et al. (2019), respectively.

2.3. Yarrowia lipolytica

Y. lipolytica is another emerging yeast RP expression host for which research interest has been steadily increasing over the past decade (Figure 1). Much like *P. pastoris*, this dimorphic yeast has been gaining popularity as a RP production host owing to its capacity to grow to high cell densities on low-cost substrates and efficient excretion of RP. Its ability to use a wide range of carbon sources including plant oils, alkanes and starch hydrolysates has further sparked industrial interest (Coelho et al., 2010; Park and Ledesma-Amaro, 2022). However, its uncontrolled dimorphism, which is not fully understood, and high oxygen requirements are limiting its industrial implementation (Madzak and Beckerich, 2013; Vandermies and Fickers, 2019). RP expression in *Y. lipolytica* is frequently done with the strong constitutive P_{TEF}. In addition, several lipid-regulated promoters have also been researched and a comprehensive review of *Y. lipolytica* promoters was recently performed by Sun et al. (2022). Major RP groups which have been produced by *Y. lipolytica* include enzymes such as lipases, proteases, phosphatases, RNases and esterases as reviewed by Darvishi Harzevili (2014). For example, First Wave BioPharma has recently issued a press release on the clinical phase trials of Adrulipase, a recombinant lipase for the treatment of exocrine pancreatic insufficiency (First Wave BioPharma, 2022; Leblond et al., 2012). An important step towards further exploitation of *Y. lipolytica* for biopharmaceutical RP production was the technology development to produce humanized glycoproteins (de Pourcq et al., 2012), patented by Oxyrane (US8026083B2 and EP2508612B1), a UK-based company focussing on enzyme therapy. Nevertheless, as compared to *P. pastoris* or *S. cerevisiae*, industrial-scale RP processes with *Y. lipolytica* are still scarce, though the steady rise in published research papers and filed patent applications in the last decade on *Y. lipolytica* indicates an upcoming increase in this area.

2.4. Kluyveromyces spp.

K. lactis is an emerging lactose-consuming yeast with high levels of protein secretion. The principal value of the *Kluyveromyces* genus lays in the fact that it is one of the few yeast genera that can utilize lactose as a sole carbon and energy source which has been particularly interesting for dairy waste stream valorization. Further owing to its high growth rate and RP yield, a wide range of enzymes, antibodies and virus particles have been produced in large-scale cultivations up to 140 m^3. Examples are the large-scale production of chymosin and β-galactosidase by DSM in the Netherlands (Guimarães et al., 2010; Spohner et al., 2016). With respect to promoter utilization for RP expression in *K. lactis*, the commonly employed *S. cerevisiae* promoters P_{GAL1} or P_{PGK} have been frequently used (Gomes et al., 2018). The lesser studied *K. marxianus* has similar beneficial properties while also being able to grow at temperatures of 45 °C and above and utilize a broad amount of substrates such as lactose, xylose, cellobiose, arabinose and polysaccharides such as pectin and inulin (Karim et al., 2020). The most important industrial applications of *K. marxianus* include the production of endogenous enzymes such as β-galactosidase, β-xylosidase, β-glucosidase, and inulase, often through the use of the *INU1* promoter (Lane and Morrissey, 2010; Zhou et al., 2018). In conclusion, this non-conventional yeast holds great potential for industrial applications and recent advancements regarding the available biotechnological tools have been reviewed by Nurcholis et al. (2020). Furthermore, the ability of *K. marxianus* to valorise waste streams is promising from an economic point of view and can aid in the transition towards a more sustainable bioeconomy.

Placeholder Figure 1.

200 **Table 1.** Yeast expression systems for recombinant protein production.

	Saccharomyces cerevisiae	*Pichia pastoris*	*Yarrowia lipolytica*	*Kluyveromyces lactis*	*Kluyveromyces marxianus*
GRAS status	Approved	Approved	Approved	Approved	Approved
Crabtree effect	Positive	Negative	Negative	Negative	Negative
Oxygen metabolism	Facultative anaerobic	Facultative anaerobic	Obligate aerobic	Obligate aerobic	Obligate aerobic
Optimal growth temperature	30-32 °C	28-30 °C	30-34 °C	25-28 °C	40 °C[a]
Native carbohydrate substrates	Hexoses Disaccharides (maltose, sucrose) Alcohols (glycerol, ethanol)	Hexoses Alcohols (glycerol, ethanol, methanol)	Hexoses Pentoses Disaccharides Alcohols (glycerol, ethanol) Lipids Alkanes	Hexoses Pentoses Disaccharides Polysaccharides Alcohols (glycerol, ethanol)	Hexoses Pentoses Disaccharides (sucrose, lactose, cellobiose) Polysaccharides Alcohols (glycerol, ethanol)
Humanized strains available	Yes	Yes	Yes	No	Yes

201 [a] high growth rates up to 47 °C have been reported (Lehnen et al., 2019)

3. Principal process parameters and scale-up considerations

The recent surge of interest in yeasts as attractive RP production hosts has led to the development of many yeast-based RP production processes for a variety of proteins. Yet, to translate these findings to a commercial scale, it is crucial to understand important scale-up parameters and the effects of the inherent fluctuating environmental conditions in large-scale fermentations and to anticipate the consequences during an early stage. Indeed, while the occurrence and impact of heterogenic zones in terms of nutrient concentrations, oxygen availability, pH, pressure, shear stress etc. are limited on a laboratory scale, on an industrial scale they often negatively impact biomass accumulation, substrate yield, productivity, and in case of more complex products such as RP, product quality. Such zones are often induced by the increased mixing time in large-scale fermentation, which can vary from less than a second at lab scale to over 100 seconds for 30 m^3 stirred tank reactors (STR) for microbial production hosts and over 1000 seconds for mammalian cells (Lara et al., 2006). While ideally processes are directly developed on a relevant scale, this is generally not possible due to restraints in the availability of equipment and the labour- and cost-intensiveness of these trials. Furthermore, when processes are scaled, this is often done by companies who are reluctant to publish their results. As a consequence, most available knowledge on scale-up is obtained through scale-down reactors (SDR), an alternative and more practical methodology to replicate the effects of large-scale fermentations in a laboratory environment (Nadal-Rey et al., 2021; Olughu et al., 2019). Here, the bioreactor setup is commonly split up into two separate parts, usually one STR and a plug flow reactor (PFR), allowing the replication of fluctuations in substrate, oxygen, or pH similar to those experienced at large-scale fermentations. Within this section, the most important process parameters and their impact on process performance are discussed in the light of scale-up considerations for yeast-based RP production based on SDR studies and available large-scale fermentation data. Alternatively, mathematical models and so-called digital twins have been used to increase scientific understanding of industrial fermentations as extensively discussed in Haringa et al. (2018) but are beyond the scope of this review.

3.1. Dissolved oxygen

The dissolved oxygen (DO) concentration has been described as the most influential parameter on overall process performance for RP production (Li et al., 2007). Indeed, for aerobic production hosts, anaerobic conditions can reduce biomass or product yield and trigger by-product formation as a consequence of metabolic shifts. In this respect, obligate aerobic yeasts such as *Y. lipolytica*, *K. lactis* and *K. marxianus* are more sensitive to anaerobic conditions as compared to *S. cerevisiae* and *P. pastoris*. Particularly for the production of cost-sensitive industrial enzymes or microbial protein, the substrate is one of the largest cost factors and optimal process efficiency is highly desirable (Puetz and Wurm, 2019). In contrast, speciality biopharmaceutical production processes have higher profit margins and are more driven by patent lifetime restraints and will thus be less inclined to intensively optimize process efficiency. Yet in this case, unforeseen by-products can negatively impact product quality and complicate downstream purification. In general and despite its increase in production cost, high oxygen availability is desired as it increases RP production, substrate consumption and biomass accumulation (Gorczyca et al., 2020; Signori et al., 2014). Furthermore, the degree of oxygen availability also impacts the morphology of *Y. lipolytica*, where high oxygen availability promotes the prevalence of an ovoid morphology over filaments (Gorczyca et al., 2020). Due to the increased shear stress and negative impact on rheology, the latter is less desired on an industrial scale. However, the low solubility of oxygen in the liquid phase, the high oxygen demand of high-cell density fermentations, and increased difficulty in supplying oxygen in large bioreactors makes it challenging to attain high oxygen availability at an industrial scale. Applicable process intensification strategies to tackle this are discussed Section 4.

In addition to the challenges of maintaining high oxygen availability, oxygen fluctuations, with zones ranging from high oxygen concentrations to microaerobic or anaerobic, are commonly encountered and may reduce the process performance of large-scale fermentations. For instance, oxygen fluctuations have been shown to reduce lipase gene expression rates for *Y. lipolytica* (Kar et al., 2010) and decrease biomass production of a *S. cerevisiae* fermentation while showing a variable effect on ethanol, acetate and glycerol accumulation (Lara et al., 2006). Lorantfy et al. (2013) observed that biomass production was reduced and by-product formation was increased as a consequence of larger oxygen fluctuations, prolonging the exposure of the baker's yeast to low DO concentrations. In essence, DO fluctuations are more difficult to control at an industrial scale as compared to DO limitations, emphasizing the importance of working with a robust production host. For instance, for *K. marxianus*, DO fluctuations between 0-20% did not notably influence the volumetric β-galactosidase production rate, biomass production or biomass yield as compared to constant DO concentrations in the same range, showing the robustness of the evaluated yeast to DO variations (Cortés et al., 2005).

3.2. Pressure

Because of the increased hydrostatic pressure in large-scale reactors (approximately 1 bar for every 10 meters of height), elevated reactor pressures are inextricably linked to large-scale fermentations. Additionally, elevated pressures of up to 1.5 barg are commonly intentionally applied to increase oxygen supply and ensure sterility. This means that microbial organisms will move through different pressure zones and potential consequences must be considered. While on the one hand, these moderately increased pressures do not lead to a notable reduction in cell viability of yeasts (Fernandes, 2005), on the other hand, an increased reactor pressure will increase the solubility of gaseous compounds in the media as a consequence of Henry's law. For instance, CO_2, the main by-product of aerobic fermentation, has been shown to affect microbial physiology as it can easily enter the cell, even more so at an elevated pressure, and lower intracellular pH. As a result of elevated CO_2 concentrations by increasing the CO_2 concentration in the ingoing gas from 0.05 to 50%, a 60% increase in maintenance energy requirements was registered for *S. cerevisiae* which would lead to a reduced biomass and product yield (Eigenstetter and Takors, 2017). Yet, another study on similarly elevated CO_2 concentrations, observed no effects on the biomass yield or viability of *S. cerevisiae* (Hakkaart et al., 2020). Here, the authors hypothesized that these contradicting results were caused by the lower growth rate applied in the latter study. Nevertheless, as many metabolic pathways are related to the CO_2 concentration, metabolic changes cannot be excluded and should be the subject of further research in a case-by-case evaluation. Nonetheless, increasing total air pressure as a strategy for process intensification has been frequently applied to meet high oxygen demands and will be more elaborately discussed in Section 4.2.2.

3.3. Substrate gradients

As a consequence of the usual single substrate addition point at the top of a reactor, substrate gradients are present during large-scale fermentations. Indeed, during a 30 m^3 fed-batch fermentation with *S. cerevisiae*, a 2.75-fold difference in substrate concentration was estimated between the addition point and the bulk medium (Larsson et al., 1996). These varying substrate concentration zones could trigger unwanted metabolic changes such as overflow metabolisms or the Crabtree effect resulting in the production of by-products. Studies evaluating the impact of increased mixing times and substrate gradients during *S. cerevisiae* cultivations have observed up to 30% lower biomass yields due to increased ethanol production (George et al., 1993; Lejeune et al., 2010; Risager Wright et al., 2016). This highlights the impact of substrate gradients on process performance and the need for further research towards large-scale production environments, where substrate costs often dictate the final product price.

3.4. pH

pH is a critical process parameter because of its impact on yeast growth, product formation, RP stability, and protease activity. Additionally, the optimal pH varies among production strains and target RPs. While in a laboratory environment buffer solutions are typically used, on an industrial scale highly concentrated acid or base solutions must be added to maintain continuous control over the pH setpoint resulting in important pH fluctuations at the point of addition. Indeed, for a pilot-scale fermentation of 100 L, a pH range of the setpoint of 4 in the bulk medium to 9 at the point of addition has been predicted (Reuss et al., 1994). Nonetheless, for *Y. lipolytica*, extended exposure to pH perturbations in a 1.6 L bioreactor affected the cell morphology but process yields and cell viability remained largely unchanged (Timoumi et al., 2017). While yeasts can grow at a wide pH range and are generally robust to fluctuations, protein quality and productivity can be negatively affected. For instance, for the production of recombinant erythropoietin by *P. pastoris*, the highest product concentration was obtained at pH 4.5 while this was reduced to less than 50% at pH 5.0 (Soyaslan and Çalik, 2011). Furthermore, excessive pH fluctuations could result in protein aggregation or degradation (Zheng and Janis, 2006). Investigating the effects of, and sensitivity to, different pH values on process performance is therefore paramount to gain early insight into the potential impact of pH fluctuations on an industrial scale.

3.5. Temperature

Process temperature plays an important role in RP productivity and product stability and quality. However, as opposed to the previously discussed parameters, no temperature gradients have been observed in an industrial-scale fermenter. This is because of the larger cooling surface, usually in the form of a cooling jacket or cooling spirals, as opposed to the single addition points of acid or base for pH control, of substrate for feeding, or bottom sparging for oxygen addition. Yet, sufficient heat dissipation can become a limiting factor during scale-up, especially for high-cell density fermentations. The heat transfer rate (HTR) can be calculated and is a measure of the cooling capability of a fermenter (Yang, 2010):

$$HTR = hA(T - T_j)$$

Where h is the heat transfer coefficient, related to the specific heat transfer efficiency of the cooling jacket material and configuration, A is the vessel jacket area, T is the culture temperature and T_j is the temperature of coolant inside the jacket. During scale-up, the ratio of jacket area A to the volume of the reactor decreases, consequently lowering cooling capacity. To overcome inadequate cooling capacity during large-scale high-cell density fermentation, process intensification strategies can be applied and are discussed throughout Section 4.

4. Process intensification strategies

Process intensification (PI) aims to improve process performance and reduce the resources required through intensified equipment or operating strategies and has been described as one of the most promising development paths to tackle techno-economic challenges and enhance industrial feasibility (Górak and Stankiewicz, 2018). As an example, the intensification of fermentation processes through *in situ* product removal can increase product titer and overall productivity for yeast-based production of industrial platform chemicals (De Brabander et al., 2021). Likewise, yeast-based RP processes often require a further increase in efficiency or productivity to compete with well-established RP production systems using *E. coli* or mammalian cells. Additionally, PI strategies can be crucial to overcome commonly observed large-scale limitations in cooling capacity or oxygen supply and improve process scalability. In recent years, multiple innovative PI strategies have been designed and evaluated on lab

scale to boost process efficiency and increase industrial feasibility. Yet, due to the often risk-averse approach of industrial processing and limited fundamental understanding of PI strategies, PI innovation is rather slow (Górak and Stankiewicz, 2018). To enhance industrial implementation of PI strategies, this review aims to increase understanding and provide a critical overview of potential PI strategies for yeast-based RP production (Table 2). Because of the well-established nature of *S. cerevisiae* and the surge of interest in *P. pastoris* for RP production, most RP-focussed PI strategies have been applied to either or both of these yeasts. However, the strategies discussed within this section could likely also be applied to other RP production hosts, but this would require further validation.

4.1. Feed-related PI strategies

The applied feed strategy has a major impact on the process performance. For the production of RP with *P. pastoris*, well-known protocols such as the ones from Pichia Protocols (Stratton et al., 1998), Invitrogen (2002) or Nature Protocols (Tolner et al., 2006) form a solid starting point for process development. Yet, the implementation of PI strategies can significantly increase process performance which is often required to attain industrially feasible production. Feed-related PI strategies that have increased process performance while also tackling scale-up challenges and limitations are discussed below.

4.1.1. Growth rate-controlled feeding

With respect to RP production, studies have shown a strong correlation between growth rate (μ) and productivity. For *P. pastoris*, this correlation was shown to be strain-dependent and the role of μ and its impact on RP production has recently been investigated (Garrigós-Martínez et al., 2019). In general, it has been shown that a reduced growth rate, as compared to μ_{max}, can significantly enhance RP productivity when working with methanol-inducible *P. pastoris* strains. Conversely, when utilizing *P. pastoris* strains that do not require methanol for RP production (e.g. P_{GAP}), a growth-coupled RP production is typically observed (Looser et al., 2014). In *S. cerevisiae,* protein-dependent correlations have been observed (Liu et al., 2013). Furthermore, growth rate control is an important factor to maintain process stability, increase reproducibility and control oxygen demands (Egli, 2015). The latter will be discussed more elaborately in the Section 4.2. Constant growth rates can be achieved by applying an exponential feed rate. To accurately and continuously control this process parameter at a fixed level and avoid deviations and time-consuming offline measurements, advanced feed rate control strategies based on online process monitoring, e.g., through detailed off-gas analysis, inline biomass probes, or mathematical models are becoming more prevalent (Lisa Mears et al., 2017; Ponte et al., 2018). For instance, for a *P. pastoris* process, controlling μ at an optimal level of 0.03 h^{-1} based on the online measurement of the ammonia consumption rate, could increase the end titer of alpha 1-antitrypsin up to 2 times as compared to conventional control strategies such as DO-stat (Tavasoli et al., 2019). For *S. cerevisiae* and *K. marxianus*, μ was accurately controlled by regulating a glucose feed based on online measurements of a biomass probe (Dabros et al., 2010; Xiong et al., 2015). In conclusion, a growth rate-controlled feed regime at a predetermined and optimized μ has been a proven strategy to enhance process performance. Hence, accurate growth rate control through process automation based on online measurement of key parameters will play a key role in the development of intensified industrial processes with improved performance and stability.

4.1.2. Mixed substrate feeding

Whereas conventional fed-batch systems use a single carbon feed for either growth or induction, a mixed feed strategy combines two or more. The initial goal of this strategy was to enhance the volumetric productivity by increasing biomass accumulation throughout the induction phase of

methanol-induced *P. pastoris* fermentations. This PI strategy has been successfully applied for both Mut^+ (Jungo et al., 2007a) and Mut^S phenotypes (Zalai et al., 2012). Doing this, a mixed feed with methanol and glycerol led to a 4.5-fold improvement in volumetric productivity of bovine lysozyme by *P. pastoris* (Brierley et al., 1990). In addition, follow-up research observed that oxygen consumption and heat production could thereby also be reduced as a consequence of the partial substitution of methanol by an alternative carbon source (Niu et al., 2013). This results from the higher oxygen requirement and heat production during methanol metabolization as compared to commonly used co-substrates such as glycerol, mannitol or sorbitol (Jungo et al., 2007a; Zalai et al., 2012). The ratio of methanol over the co-substrate in the mixed feed plays an important role in RP productivity. While at suboptimal ratios RP productivity dropped to zero, for an optimized ratio RP productivity could be increased by more than 40% compared to a methanol-only benchmark (Niu et al., 2013). However, because of the additional feeds, process complexity will increase, undesired protein variation could be introduced (Ye et al., 2011), and repression of the induction system has been described, thereby lowering process efficiency (J. Wang et al., 2017). Yet, the latter has been circumvented by using non-repressive carbon sources such as sorbitol, mannitol or ascorbic acid (Vuree, 2020; Zavec et al., 2020). As an example, a methanol/sorbitol mixed feed strategy with *P. pastoris* reduced heat production and oxygen consumption by almost 40% while increasing RP productivity by 30% as compared to the conventional process with a sole methanol feed (Jungo et al., 2007b). In conclusion, for the widely used methanol-inducible *P. pastoris* processes, a mixed feed strategy could be an attractive PI strategy to lower oxygen and cooling demands but requires careful optimization of all carbon fractions in the feed to avoid a reduction in RP productivity. Additionally, the associated increased substrate cost should be justified by, on the one hand, a proportional increase in RP formation or, on the other hand, a reduction in costs related to cooling or oxygen supply.

4.1.3. Intermittent feeding

An intermittent feeding strategy applies successive substrate feeding breaks during which the yeast is deprived of any carbon source. As a response to these applied stress conditions, higher RP synthesis rates have been observed for P_{GAP}-controlled *P. pastoris* processes. It has been hypothesized that this increase could be caused by the upregulation of glycolytic pathways (Garcia-Ortega et al., 2016). By optimizing the duration of the carbon starvation periods, this rather simple to implement fed-batch strategy has led to an increased biomass-specific yield and could increase the substrate yield by 50% while overall process productivity could be increased by 30% (Garcia-Ortega et al., 2016). Because of the higher biomass-specific yield, lower biomass concentrations could be targeted while still maintaining similar end product concentrations which is beneficial for lowering oxygen demand and cooling requirements. However, repeated carbon starvation cycles are known to induce stress on the production organism and consequentially lower RP yield or quality due to proteolytic degradation (Heo et al., 2008). Nevertheless, this readily scalable strategy appears promising for large-scale fermentation processes owing to the increased cost-effectiveness following the higher substrate yield. To date, industrial feasibility of the strategy has not been investigated and further research is necessary to increase insight into the consequences of repeated carbon starvation cycles on protein breakdown and evaluate the effectiveness of this strategy for other types of RP as well as production hosts.

4.2. Oxygen-related PI strategies

One of the most commonly observed challenges during scale-up involves insufficient oxygen availability for the cells. Indeed, as discussed in Section 3.1, the oxygen concentration and its fluctuations in a bioreactor can significantly influence process performance. To overcome these limitations, PI strategies aiming to increase the oxygen transfer or lower oxygen requirements can be applied, hereby increasing technical feasibility as well as process performance. Alternatively, process

temperature, choice of the production host, promoter, and strain phenotype can also considerably influence oxygen requirements, which will be more elaborately discussed in the next sections.

4.2.1. Lowering growth rate

A drawback of high-cell density fermentations, as commonly observed with yeast, is the high OTR requirement. As discussed above, the applied growth rate is known to control oxygen demands. While generally regarded as disadvantageous due to the increased processing time and lower RP productivity, operating at a lower cell growth rate by limiting the substrate feed rate is an interesting strategy for RP production (Looser et al., 2014). The reduced cell growth results in lower oxygen and maintenance energy requirements, thereby increasing cell viability and minimizing cell lysis and the consequential release of intracellular proteases into the medium causing RP product breakdown (Zhao et al., 2008). Therefore, low growth rates could allow extended accumulation of the desired proteins and has successfully been applied on pilot-scale. As an example, limiting the growth rate of a P_{GAP}-controlled *P. pastoris* strain resulted in a 13% increase in specific lipase activity and over 50% reduction of the cell maintenance energy consumption upon scale-up to 800 L. However, as a consequence of lower biomass accumulation, lipase productivity was decreased by about 10% (Zhao et al., 2008). Nevertheless, the accumulation of 20% less biomass is expected to reduce downstream purification costs.

4.2.2. Increasing total air pressure

Oxygen limitation can also be avoided by increasing the oxygen transfer rate (OTR). The OTR is a measure of the rate at which oxygen is transferred from a gaseous phase (e.g. air) to a liquid phase (e.g. cultivation medium) and can be optimized by reactor type and design, which has been extensively reviewed elsewhere (Garcia-Ochoa and Gomez, 2009). Yet, most advanced bioreactor types able to achieve high OTR values are still within a research stage. Alternatively, the OTR can be improved by increasing the partial pressure of oxygen through the supply of oxygen-enriched air, or by increasing the total reactor pressure. While in a laboratory environment, further increase of the oxygen supply after maximizing both stirrer speed and aeration rate is often achieved through pure oxygen supplementation, the operational risks and high cost of pure oxygen limit this strategy for large-scale fermentations. Contrastingly, increasing the total air pressure is a viable and well-known strategy for industrial fermentations which are commonly performed in pressure-resistant stainless-steel reactors (Knoll et al., 2007). Because of the higher solubility of oxygen in the medium phase at an increased pressure, pure oxygen supplementation can hereby be avoided (Liu et al., 2016). As an example, an enhanced oxygen uptake rate by increasing the reactor pressure from 0.2 to 0.9 barg resulted in a 50% increased β-glucosidase yield for a *P. pastoris* fermentation process (Charoenrat et al., 2006). Also, by increasing the operating pressure up to 7 barg for a *Y. lipolytica* process, lipase production was increased by over 500% (Lopes et al., 2008). Yet, a pressure above approx. 1.7 barg requires specialized equipment, further increasing capital investments.

4.2.3. Hypoxic conditions

The driving force of oxygen transfer to the medium phase can be increased by lowering the DO setpoint (e.g., < 5%) (Charoenrat et al., 2005). As a result, a reduced amount of energy in the form of agitation or aeration is required for similar oxygen demands which is beneficial from both a technical and economic point of view. However, under these low oxygen or hypoxic conditions, varying results have been obtained depending on the specific yeast species. By reducing DO of a *P. pastoris* fermentation process, a 2.3-fold higher volumetric RP productivity could be attained and product purity could be increased because of a reduced amount of homogeneous proteins secreted in the medium (Baumann et al., 2008; Garcia-Ortega et al., 2017; Gasset et al., 2022). Even more, lower biomass accumulation

was observed, which facilitates cell removal during downstream processing. Contrastingly, for *S. cerevisiae* RP productivity was lowered and cell growth was negatively affected (Aon et al., 2018; Baumann et al., 2011). Besides, monitoring and controlling the desired hypoxic conditions is challenging. A commonly applied strategy for hypoxic process control involves monitoring the specific ethanol production rate. Yet, this requires intensive sampling and off-line quantification of the ethanol concentration in the culture medium. Alternatively, respiratory quotient (RQ) monitoring, based on O_2 and CO_2 data from off-gas analysis which indicates the physiological state of the yeast, has been used as a continuous monitoring method for the degree of oxygen limitation (Gasset et al., 2022).

4.3. Continuous cultivation

Progressing towards continuous production has led to an increased process efficiency and subsequent improved cost-competitiveness within several industries. For industrial yeast-based RP production, continuous cultivation presents a highly promising PI strategy to improve its industrial feasibility as it allows extension of the production phase, thereby reducing process downtime and increasing space-time yield. Increased product consistency and manufacturing flexibility as well as equipment size reduction are some of the major cost reduction factors that can be obtained through continuous processing (Peebo and Neubauer, 2018; Walther et al., 2015). Furthermore, higher RP yields have been obtained through continuous cultivation, which could be attributed to the higher fraction of younger, more efficient cells as compared to traditional batch or fed batch processes (Nieto-Taype et al., 2020) or the lower stress levels observed in these cells (Zahrl et al., 2017). For example, a recent *P. pastoris* study demonstrated that within a timeframe of six weeks, almost six times more recombinant lipase B could be produced in a continuous mode as compared to a traditional fed-batch mode, thereby enabling lower production costs (de Macedo Robert et al., 2019). Another *P. pastoris* study was able to increase the volumetric productivity for a recombinant Hepatitis B antigen by roughly 50% by employing a continuous production mode. The authors reported high genetic stability and the absence of contaminants after two weeks of cultivation (Rahimi et al., 2019). Through the continuous cultivation of a β-galactosidase enzyme using a lactose-consuming *S. cerevisiae* strain, productivity was increased up to 11-fold as compared to the batch benchmark process (Domingues et al., 2005).

Yet, continuous cultivation often involves increased complexity for process control, requires high genetic stability of the production strain, is more prone to contamination, and faces regulatory challenges with regard to the production of biopharmaceutical RP (Fisher et al., 2019). For the latter, additional challenges are observed as compared to industrial enzymes due to their typically lower production quantities, the increased importance of time to market and the required time for regulatory approval. Yet, with regard to regulatory approval, the rise of continuous (perfusion) cultivations for biopharmaceutical RP production with mammalian cell cultures could also accelerate the transition towards yeast-based continuous cultivation (L. Mears et al., 2017). Currently, continuous industrial production of insulin, one of the first produced RP in yeast, is being performed by Novo Nordisk using *S. cerevisiae* (Peebo and Neubauer, 2018). To the best of our knowledge, no reports on commercial continuous RP production have been described for the other yeasts discussed within this review, rendering fed-batch processing still the most prevalent mode of operation to date. The lack of such processes could be caused by the low risk tolerance, short process development times, the relatively young industry of biotechnology and the initial focus on the development of innovative RP rather than PI and cost reduction strategies (Cankorur-Cetinkaya et al., 2018; Walther et al., 2015). Yet, the pressure to continuously lower production costs of RP will push researchers to tackle current challenges of continuous yeast-based cultivation and it is expected that with the maturation of the biotechnology industry, the implementation of continuous processes on an industrial scale will increase.

4.4. Lowering process temperature

Lowering the process temperature setpoint is a convenient PI strategy that has led to 1) reduced proteolytic degradation, 2) increased product quality, and 3) increased protein productivity. The former two can be explained by reduced thermodynamic protease activity at lower temperatures and reduced release of intracellular proteases into the medium as a consequence of decreased metabolic stress or cell death (Jahic et al., 2003b). Lower temperatures can furthermore increase protein stability thereby reducing protein refolding requirements and consequently increasing RP productivity (Dragosits et al., 2009). For example, Jahic et al. (2003b) could double the final RP concentration, yet this required the temperature to be lowered to 12 °C as compared to 30 °C. Other studies have reported specific productivity improvements of up to 100-fold by lowering the temperature to 20 °C (Dragosits et al., 2009; Jin et al., 2011; Li et al., 2013; Zhong et al., 2014). Additionally, at lower temperatures oxygen demand is lowered because of a reduced metabolic activity, while oxygen solubility is increased (Trentmann et al., 2004). However, a temperature close to optimal is desired to maximize the growth rate of the yeast and, as a consequence, the abovementioned benefits are usually at a cost of a lower growth rate. Furthermore, care must be taken when scaling these processes since sufficient heat dissipation will become more challenging and cooling costs will rise with an increasing bioreactor size. This especially holds for high-cell density fermentations and those with high heat generation such as methanol-induced *P. pastoris* fermentations. Yet, other PI strategies can be applied in parallel to lower heat production and decrease oxygen requirements (Table 2). Hence, during process development an important trade-off must be made in which increased protein production and/or quality is balanced with higher cooling capacity requirements and their related processing costs while also considering the technical limitations of a large-scale bioreactor.

4.5. Lowering process pH

Similar to temperature, the pH can be optimized to reduce proteolytic degradation and increase overall process efficiency (Kong et al., 2009). In general, conditions of low pH reduce proteolytic activity because of the associated partial or complete inactivation of proteases (Jahic et al., 2003a). While in contrast to lowering the temperature, adjusting pH is not limited by technical limitations. Yet, strain- and protein-specific limitations will apply and must be accounted for. This strategy was found effective for the production several types of RP including a cellulose-binding module fused to a *Candida antarctica* lipase (CBM-CALB) where the fraction of full-length protein was increased from 40 to 90% by decreasing the pH from 5.0 to 4.0 (Jahic et al., 2003a), and a proteolysis reduction of a growth hormone (IGF-1) by lowering the pH to 3.0 (Baghban et al., 2019). Additionally, processes at a lower pH setpoint are less prone to contamination.

4.6. Strain selection and development

Besides process-related intensification strategies, strain selection and engineering can significantly improve process performance and technical feasibility. While an in-depth review of strain development is beyond the scope of this review, some prominent examples are provided. For instance, the use of thermotolerant organisms, such as *K. marxianus*, can improve cooling efficiency because of the increased driving force between the coolant and fermentation medium, thereby lowering process costs related to temperature control (Gombert et al., 2016). A higher temperature furthermore reduces the risk of contamination. Yet, the optimal temperature setpoint will also be determined by the stability of the target protein at these elevated temperatures. Alternatively, strain phenotypes have been developed with reduced heat generation. For instance, for *P. pastoris*, this has led to the development of strains where both *AOX1* and *AOX2* genes are knocked out, resulting in a Mut^- strain. Recent progress has shown that important scale-up parameters including the oxygen demand, heat

output and specific methanol uptake rate were reduced by more than 80% as compared to the more commonly used Mut^S or Mut^+ alternatives, while high viability could be maintained throughout the fermentation (Zavec et al., 2020). Additionally, because of the lower methanol uptake rate, which was 93% lower as compared to a Mut^S strain, lower volumes of methanol are required and hence the inherent drawbacks of having to store large amounts of methanol can be minimized, while also lowering feedstock costs. An important drawback is the currently lower (42%) product titer as compared to a Mut^S phenotype. Yet, while still in a research stage, new methanol-free promoter systems are continuously being developed aiming to lower heat production and oxygen demand (Garrigós-Martínez et al., 2021). As an alternative to lowering oxygen demand, the oxygen uptake rate (OUR) in yeasts can be enhanced through modification of the cell physiology. This has been demonstrated in *Y. lipolytica* and *P. pastoris* through co-expression of *Vitreoscilla* hemoglobin, resulting in nearly a two-fold increase in the activity of a recombinant lipase produced using *P. pastoris* (Bhave and Chattoo, 2003; Wang et al., 2012b). Finally, recent advancements in synthetic biology techniques, including the latest CRISPR/Cas9 technology, are expected to play a major role in improving homologous recombination efficiency, protein folding, and optimizing promoters, terminators, and transcription factors. For a more comprehensive view on the subject, the reader is referred to recent reviews (Pan et al., 2022; Patra et al., 2021; Raschmanová et al., 2021, 2018).

582 **Table 2.** Process intensification strategies to overcome principal scale-up challenges.

Scale-up challenge	Process strategies
Oxygen limitation	**Increase oxygen supply by** Reactor design Increasing total operating air pressure Lowering the DO setpoint to increase the driving force Lowering the temperature to increase the oxygen solubility Enhancing OUR through cell physiology modification **Reduce oxygen demand by** Lowering feed rate to maintain lower growth rate Mixed feed strategies Intermittent feeding strategy Lower temperature setpoint to reduce metabolic activity Development of alternative promoter systems
Insufficient cooling capacity	**Increase cooling efficiency by** Increasing temperature setpoint Reactor design **Reduce heat production by** Reducing working biomass concentration Applying a mixed feed strategy Applying an intermittent feeding Development of alternative promoter systems (e.g., Mut^-) Using alternative production hosts
Gradients (e.g., substrate, pH)	Optimize reactor design to reduce gradients Select robust strains to reduce impact of gradients
Low or inconsistent protein quality	**Reduce proteolytic degradation by** Lowering the temperature to avoid cell lysis and release of protease to the medium Lowering the temperature to reduce protease activity Lowering pH setpoint to reduce protease activity Developing protease deficient strains **Reduce batch-to-batch variability by** Implementing a continuous cultivation strategy Developing protease deficient strains Advanced process automatization with online monitoring and process control

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5. Conclusions and prospects

Yeasts have demonstrated high potential as industrial hosts for RP production. While *S. cerevisiae* and *P. pastoris* have been used to produce a variety of RP products at an industrial level, including microbial protein, enzymes and biopharmaceutical proteins, research efforts towards the use of alternative or non-conventional yeasts such as *Y. lipolytica*, *K. lactis* and *K. marxianus* have been increasing because of the ever-rising importance to use sustainable and low-cost feedstocks. The higher protein secretion efficiency of yeasts as compared to bacterial production hosts allows to significantly reduce the downstream processing costs of price-sensitive products, hereby attaining economically feasible production. In addition, particularly for the production of biopharmaceutical RP, yeasts provide a fast development timeline and can use cheap cultivation media while being able to produce complex humanized proteins.

Yet, the road from laboratory-scale development to an industrial process is challenging and starts with the selection of a suitable production host. In addition to the widely used selection criterium of maximal productivity, the strain robustness towards the conditions of large-scale fermentations must be considered and investigated during early process development. Indeed, in large-scale bioreactors, fluctuations in terms of dissolved gaseous compounds, substrate concentrations, and pH, are not easily controlled or overcome and hence their impact on the process performance should be taken into account when scaling a fermentative production process.

Besides gradients, technical limitations of large-scale bioreactors are often the cause of commonly encountered scale-up challenges such as insufficient oxygen supply or cooling capacity and must be considered during the early stages of process development to establish crucial process boundaries. To overcome these challenges and expand the feasibility window for process operation, multiple effective process intensification strategies focussing on lowering oxygen demands and cooling requirements while increasing product quality and reducing process costs have been investigated to enhance industrial feasibility and subsequent commercialization. In addition, strain engineering efforts and the development of novel promoter systems for *P. pastoris* could bring further process improvements, the latter through the use of non-hazardous inducers with concurrent reduced oxygen demand or heat production.

Finally, up until now, most research has focused on the use of *S. cerevisiae* and *P. pastoris* for RP production, both from a strain engineering and fermentation process development point-of-view and has already allowed efficient industrial yeast-based RP production. Nonetheless, future process development and intensification efforts should also be directed towards other emerging yeast species, opening doors to a wide range of potential novel RP products with a variety of applications and to the use of alternative abundant, economical and sustainable feedstocks.

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