Optimising yeast as a host for recombinant protein production (review)

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Abstract

Having access to suitably stable, functional recombinant protein samples underpins diverse academic and industrial research efforts to understand the workings of the cell in health and disease. Synthesising a protein in recombinant host cells typically allows the isolation of the pure protein in quantities much higher than those found in the protein's native source. Yeast is a popular host as it is a eukaryote with similar synthetic machinery to the native human source cells of many proteins of interest, whilst also being quick, easy and cheap to grow and process. Even in these cells the production of some proteins can be plagued by low functional yields. We have identified molecular mechanisms and culture parameters underpinning high yields and have consolidated our findings to engineer improved yeast cell factories. In this chapter we provide an overview of the opportunities available to improve yeast as a host system for recombinant protein production.

Key Words: recombinant protein production; yeast; strain engineering; bioprocess control

1. Introduction

The development of recombinant protein production hosts that can be used to produce a wide range of targets is a key area of research (***1***). This is particularly true for the production of membrane proteins, which are high value targets in the drug discovery pipeline, and which cannot yet be produced in high yields in a predictable manner (*2, 3*). Yeast species, especially *Pichia pastoris* and *Saccharomyces cerevisiae* **(*2, 4-6***) have already been identified as one of the most important components of a matrix of protein production hosts (***7***), and have contributed a substantial number of functional (***8***) recombinant eukaryotic membrane proteins in very high yields (e.g. (***9***)) thus enabling high resolution structure determination (***10-13***).

Yeasts are well-characterised organisms and can be cultured very cheaply and easily in large quantities. They are also straightforward to manipulate genetically: publicly-available sequences are available for both *P. pastoris* (***14***) and *S. cerevisiae* (***15***). As yeasts are eukaryotes, they have protein-processing and post-translational modification mechanisms related to those found in mammalian cells. However, they contain ergosterol rather than cholesterol in their membranes and recombinant proteins produced using yeast may be decorated with high-mannose type sugars, which are not native to mammalian cells (***2***). Fortunately these problems have been overcome, in part, through strain engineering (*16, 17*). Despite these potential limitations, recombinant pharmaceuticals including insulin (***18***) and several vaccines (e.g. (***19***)) have all been produced in yeasts for commercial use, demonstrating their importance to the pharmaceutical industry. In order to optimise their productivity, two complimentary approaches have been taken: engineering the yeast strain and modifying the culture or 'bioprocess' conditions. Both are discussed in this chapter and are exemplified by studies on *S. cerevisiae*.

2. Host cell engineering to increase recombinant protein yields

Strategies to increase the recombinant protein productivity of host cells have been examined in both prokaryotic and eukaryotic cells. For example, a strain of *Escherichia coli* with high cardiolipin content (14% compared with 3% in wild-type strains) has been found to produce increased yields of some proteins (*20, 21*). *Lactococcus lactis* has also been evolved to generate strains with modest improvements in their yield properties (***22***). In both cases mutations in the promoter system, and not in any *in vivo* cellular components of the translation or folding machinery, were found to have slowed the protein synthesis rate, presumably allowing the newly-synthesised protein to be accommodated into the endoplasmic reticulum with a minimum of inclusion body formation and cell death.

We and others have used a more systematic approach (***23-25***) to optimise a host cell for recombinant protein production, with the specific goal of understanding the molecular barriers to achieving high yields. We therefore looked at the global host cell response to producing a membrane protein in *S. cerevisiae* (***23***). Using transcriptome arrays, we examined two different growth conditions that both led to relatively low protein yields (compared with normal conditions) and looked for changes in mRNAs that occurred in the same direction in both sets. This highlighted a set of genes, which was validated against those identified when comparing the single high-yielding growth condition with normal conditions. Genes that were down-regulated under low-yielding conditions were up-regulated under high-yielding conditions and *vice versa* (***26***). This allowed us to identify genes that influenced the yield per cell (***27***): three are known components of the transcriptional SAGA (*GCN5* and *SPT3*) and mediator (*SRB5*) complexes, whilst a fourth, *BMS1*, is involved in ribosome biogenesis (***26***). Interestingly, there was an increase in *BMS1* transcript number compared to wild-type in all our high-yielding host strains. In particular, we were able to tune the expression of *BMS1* to maximise yields (***26***).

Work such as this has highlighted other relevant pathways for further study. For example in producing a recombinant membrane protein, there may be a benefit in altering the membrane lipid content in the host cell (***25***), as has been seen previously for *E. coli* strains (*20, 21*). Opi1 protein acts as a transcriptional suppressor, affecting the synthesis of phospholipids (***28***). An *opi1Δ* strain therefore contains increased phospholipid concentrations compared with wild-type strains (***29***). A study of strains with altered lipid composition in our own laboratory (e.g. *opi1Δ*, *bio2Δ* and *erg6Δ*) revealed that *opi1Δ* yielded 8 times more protein than the corresponding wild-type strain (unpublished). Similar studies in *P. pastoris* on the

global response to producing a recombinant protein (e.g. (***30***)) should soon lead to similar advances.

3. Optimising culture conditions to increase recombinant protein yields

Although the potential returns are high, engineering a host organism to increase recombinant protein yields can be expensive and time-consuming, especially if strategies are based on transcriptome analysis. An alternative, complimentary and potentially cheaper route is to optimise the experimental culture conditions, ideally using a statistical design of experiments approach (see **Chapter 11**). The development of growth media for mammalian cell culture has led the way in this area. For example, medium composition has been key to maintaining viable, high density cultures to obtain increased monoclonal antibody titres (***31***) or to be able to use chemically-defined serum-free medium (*32, 33*).

Variations in the composition of the culture medium, its pH, its temperature, the availability of dissolved oxygen and the details of the induction regime (e.g. concentration of inducer, as well as the point and duration of induction) may all lead to improvements in the productivity of yeast cultures. For example, the yield of active G protein-coupled receptors (GPCRs) produced in *P. pastoris* was increased in over half the cases when the growth medium was supplemented with 2.5% dimethyl sulfoxide (DMSO; 16 out of 20) or 0.04 mg mL^{-1} L-histidine (12 out of 20) (***34***). However, in another study, the human tetraspanin protein, CD81, was produced and purified from *P. pastoris* (***35***), but supplementation with 2.5% DMSO did not increase yields (unpublished). Often, however, the induction regime in *P. pastoris* has a much greater effect on yield than the growth conditions ***(36)***.

For cultivating *S. cerevisiae*, the most commonly-used media formulations in our laboratory are CBS (devised by the Centralbureau voor Schimmelcultures, the Netherlands; (***37***)) and those based on YNB (Yeast Nitrogen Base; (***38***)). YNB-based media are simpler to make than CBS, and originate from the work of Burkholder and Wickermann who used alterations in growth media to discriminate between yeast strains (***39-41***). For use with typical auxotrophic laboratory strains, both formulations require additional supplementation with an amino acid mixture, adenine and uracil (e.g. complete supplement mixture or CSM), as well as a carbon source (see **Notes 1 and 2**). Growth to medium cell densities (OD_{600} < 50) is possible in 2 × CBS medium supplemented with 20 or 50 g L^{-1} glucose as the carbon source (*42*, *43*), while growth in a YNB-based medium typically yields less biomass and thus less recombinant protein. The use of alternative carbon sources, such as ethanol, has also been a route to increased protein yields (***44***). Overall, CBS medium supports a higher growth rate than YNB-based medium, but is much more labour intensive to prepare (***23***).

[Insert Table 1 here]

It has become clear in our own studies that YNB-based media are not ideal for protein production in *S. cerevisiae* (***25***). In order to combine the simplicity of the YNB-based formulation (see **Note 1**) with the improved performance of CBS medium (see **Note 2**), we have undertaken further optimisation studies. For example, since the membranes of the endoplasmic reticulum have a high content of phosphoinositol lipids, we examined the effect of supplementing a YNB-based medium with myo-inositol to the levels found in CBS (**Table 1**) on recombinant membrane protein yield. Initial data suggesting that yields were improved (unpublished) are consistent with our previous observations for the *opi1Δ* strain, above, as well as literature reports suggesting that myo-inositol is essential for relieving some cellular

stresses caused by recombinant membrane protein production (***45***). Furthermore, supplementation with myo-inositol (as well as biotin; **Table 1**) was found to increase growth rates to values typical of CBS medium. Adding both myo-inositol and biotin also increased the growth rate, but less dramatically (**Figure 1**). Addition of cobalt was found to decrease the growth rate even in the presence of myo-inositol and biotin, although in the presence of copper this decrease did not occur. Observations such as these could be followed up using a statistical design (***46-48***) to minimise the number of experiments required to examine all possible combinations of these medium components; especially as metal ion requirements for *S. cerevisiae* are often strain specific (***49***) (see **Chapter 11**).

[Insert Figure 1 here]

The beneficial effect of adding myo-inositol to YNB-based media on recombinant membrane protein yield may indicate that different medium components could be fine-tuned to relieve the stresses induced during recombinant soluble protein expression (***50***). For example induction of the unfolded protein response (***51***) might require a different balance of nutrient components. Adding only the required nutrients at just the right concentration for maximum productivity should enable this to be achieved in the most cost- and time-effective manner.

4. Conclusions

YNB-based media are quick and easy to make, but do not support optimal recombinant protein yields. Supplementing the formulation with additional myo-inositol and biotin may overcome these limitations. This is especially true for recombinant membrane proteins.

5. Notes

1. A YNB-based medium for a strain with a given auxotrophy is composed of 1.7 g L^{-1} YNB (without ammonium sulphate and amino acids, BIO101 #4027-012), 5 g L^{-1} ammonium sulphate and CSM minus the relevant component, as directed by the manufacturer (BIO101 #4510-333). Glucose is autoclaved separately as a 20% stock solution and added to a final concentration of 2%. If the glucose is autoclaved together with the ammonium sulphate and YNB, the solution will have a yellowish appearance. At a final OD_{600} <15 this will not affect growth, but it is quite probable that nutrient limitation events will occur when growing to higher densities (using e.g. 5% glucose), due to glucose-induced reactions during autoclaving of the nutrients together. It is generally considered that the maximum glucose concentration that a YNB-based medium will support is 5%; at higher glucose concentrations, for example in a fed-batch culture, additional nutrients should be provided.
2. 2 × CBS medium is composed of 10 g L^{-1} ammonium sulphate, 6 g L^{-1} potassium dihydrogen phosphate, 1 g L^{-1} magnesium sulphate heptahydrate plus twice the amount of CSM minus the relevant component directed (BIO101 #4510-333). Glucose is autoclaved separately as a 20% stock solution and added to a final concentration of 2%. Finally, 2 mL L^{-1} each of trace element solution and vitamin stock solution are added. 1 L trace element solution is composed of the following: 15 g EDTA, 4.5 g $ZnSO_4 \cdot 7H_20$, 1 g $MnCl_2 \cdot 4H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 0.3 g $CuSO_4 \cdot 5H_2O$, 0.4 g $Na_2MoO_4 \cdot 2H_2O$, 4.5 g $CaCl_2 \cdot 2H_2O$, 3g $FeSO_4 \cdot 7H_2O$, 1g H_3BO_3 and 0.1 g KI. The pH is maintained at 6.0 with 1 M NaOH throughout the addition and finally adjusted to pH 4 with 1 M HCl prior to autoclave sterilisation and storage at 4°C in the dark. 1 L vitamin solution is composed of the following: 0.05 g biotin, 1 g calcium D-panthothenate, 1 g nicotinic acid, 25 g myo-inositol, 1 g thiamine hydrochloride, 1 g pyridoxol hydrochloride and 0.2 g D-

amino benzoic acid. The pH is maintained at 6.5 with 1 M HCl. The vitamin solution is filter-sterilised and stored as 20 mL aliquots at 4°C.

6. Acknowledgements

This work was supported by the European Commission via contract LSHG-CT-2004-504601 (E-MeP), LSHG-CT-2006-037793 (OptiCryst) and Grant 201924 (EDICT) to RMB. The BBSRC supports bioreactors and a flow microcalorimeter in the RMB Laboratory through an REI award.

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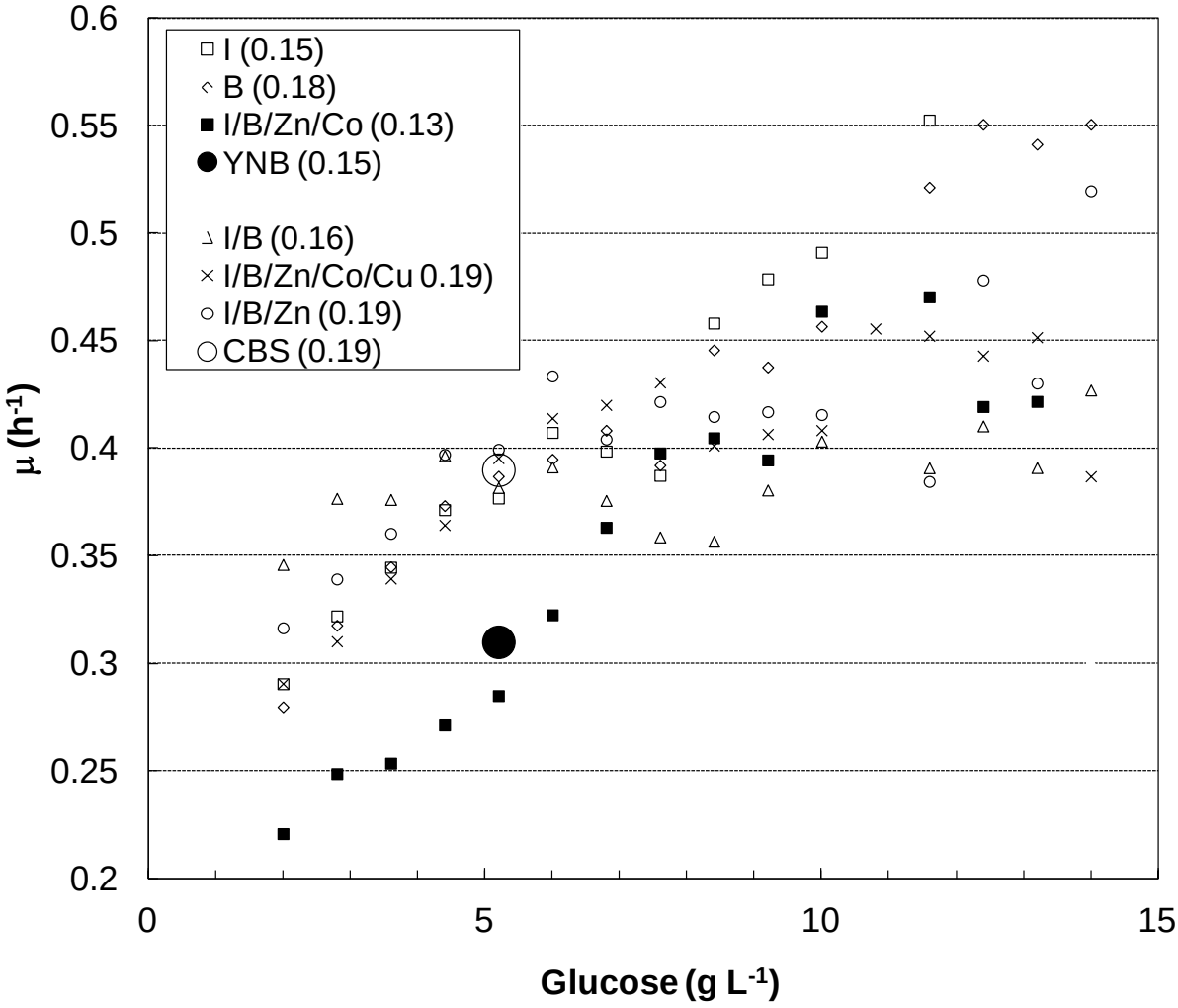
Figure legend

Figure 1: Specific growth rates for *S. cerevisiae* strain BY4741 producing recombinant Fps1. The specific growth rate (μ; h^{-1}) was computed using the amount of base added in two adjacent 20 min segments in the 2-14 g L^{-1} residual glucose range (***23***). The average dry weight after consumption of the carbon source (at 55 h) was 0.17g biomass (g glucose)$^{-1}$. This value increased by 0.04 g g^{-1} in the presence of myo-inositol and biotin. Media additions to YNB are as follows: myo-inositol (open squares); biotin (open diamonds); myo-inositol/ biotin/zinc/cobalt (filled squares); myo-inositol/biotin (open triangles); myo-inositol/biotin/zinc/cobalt/copper (crosses); myo-inositol/ biotin/zinc (open circles). YNB alone is shown as a large filled circle. 2 × CBS is shown as a large open circle. Individual dry weight values (g g^{-1}) are listed in parenthesis after each condition (n=2; standard deviation < 0.008 for all data points).

Table 1: Comparison of selected nutrient components in media based on CBS and YNB formulations.

Nutrient	**CBS (mg L^{-1})**	**YNB (mg L^{-1})**	**Factor difference (CBS:YNB)**
Biotin	0.05	0.002	25
myo-inositol	25	2	12.5
Cobalt chloride hexahydrate	0.3	0	∞
Zinc sulphate heptahydrate	4.5	0.4	11.25
Copper sulphate pentahydrate	0.3	0.062	7.5

S. cerevisiae requires a minimum concentration of Cu^{2+}, Zn^{2+} and Fe^{2+} of 15, 200 and 150 μg L^{-1}, respectively (***49***). In YNB-based media these concentrations are typically 16, 91 and 88 μg L^{-1}, respectively, and in CBS they are 76, 1,023 and 602 μg L^{-1}, respectively. Other metal ions not included in the YNB formulation are Co^{2+} and Ni^{2+}. Cobalt in particular is known to have an inhibitory effect on growth (***52***). Boric acid is present in YNB-based formulations at a concentration of 500 μg L^{-1} (8μM) and is known to increase biomass yields during respiratory growth at 150 μM (***53***). The five nutrients with the largest difference in concentration between CBS and YNB media are listed.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10
15
Glucose (g L-1)
I (0.15)
B (0.18)
I/B/Zn/Co (0.13)
YNB (0.15)
I/B (0.16)
I/B/Zn/Co/Cu 0.19)
I/B/Zn (0.19)
CBS (0.19)