Animal-free media optimization for recombinant protein production in Yeast

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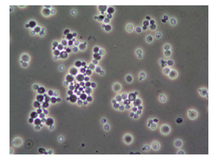

Introduction

Yeast are fast becoming one of the most widely used hosts for the production of recombinant proteins. Yeasts have similar positive growth characteristics to that of traditional prokaryotic hosts, with the added benefit of possessing a secretory pathway resulting in the glycosylation of the target protein, a characteristic normally present in slow growing mammalian cells.

Although yeasts can be grown in both complex and synthetic media, complex media can have a number of benefits related to recombinant protein fermentations including increased biomass and improved product quantity and quality. In recent years there has been a growing trend towards the use of non-animal media components in an effort to remove the risk of contamination of raw material with adventitious agents.

The purpose of this study was to optimize animal component free (ACF) complex media formulations for recombinant protein production in yeast. Three of the most popular yeasts used for the production of recombinant proteins are S*accharomyces cervesiae, Pichia pastoris and Hansenula polymorpha* and a number of strains from these organisms were used in this study.

Materials & Methods

Pre-screening for growth:
Three *Pichia pastoris* strains, two *Hansenula polymorpha* strains and two *Saccharomyces cervesiae* strains were screened for growth response to a combination of Kerry's animal component free protein hydrolysates and yeast extracts. The *Pichia* & *Hansenula* strains were grown in a BMGY media containing 30 g/l nitrogen source, 1.34 % YNB, 100 mM potassium phosphate buffer, 3 % glycerol and 40 µg/l biotin. Growth was measured by absorbance at an O.D. of 620 nm. Experiments were performed in 96 well plates and were placed in an incubator set to 30 C with agitation at 300 rpm for 18 hours.
The Saccharomyces strains were grown in medium containing 30 g/l nitrogen source, 1.34 % YNB, 100 mM potassium phosphate buffer and 32 ug/l biotin. Growth was measured by absorbance at an O.D. of 620 nm. Experiments were performed in 96 well plates and were placed in an incubator set to 30 C with agitation at 250 rpm for 27 hours.
Extracellular expression of protein in *Pichia Pastoris*:
The five most promising animal ACF samples that boosted the performance of *Pichia pastoris* were reanalysed in two proprietary *Pichia* strains already in use in industry to assess their effects on protein quantity and quality by comparing protein samples produced in ACF media versus animal based media. Protein analysis was performed by SDS PAGE.
The growth of the first strain involved inoculating a single colony into 100 ml YPD medium for 24 hrs at 250 rpm. 0.5 ml of this inoculum was added to 100ml BMGY containing 40 g/l nitrogen source, 100 mM potassium phosphate buffer, 13.4 g/l YNB, 40 µg/l biotin and 4 % glycerol. Flasks were incubated at a temperature of 29 C and agitated at 250rpm. At 24, 48 and 72 hours methanol was added to a final concentration of 4 %. The growth of the second *Pichia* strain was as previously described, except that after the initial growth phase in BMGY media (24 hours), the cells were centrifuged and resuspended in fresh 10 ml BMMY media (40 g/l nitrogen source, 14.82 mM potassium phosphate buffer, 13.4 g/l YNB, 40 µg/l biotin and 4 % methanol) and left overnight. The following morning, a methanol/nitrogen source feed was added to a final concentration of 4 % methanol/40 g/l nitrogen source. This was repeated twice daily for two days.
Extracellular expression of protein in Saccharomyces cerevisiae:
A *Saccharomyces* strain was also analyzed with regard to extracellular-expressed protein quality and quantity. The strain was inoculated in YEG media and incubated at 30 C with agitation at 250 rpm for 7 hours. This was then used to inoculate (2 %) a range of Kerry ACF media along with the ACF reference. The production media consisted of 50 mls media containing 10 g/l nitrogen source, 13.4 g/l YNB, 21 g/l glucose and 0.2 g/l MgSo4 which was incubated at 30 C with an agitation of 250rpm.
Activity of intracellularly-expressed β-galactosidase:
To analyze the effect of the ACF media on protein activity, a β-galactosidase assay was performed using a *Pichia pastoris* GS115 β-gal strain (Invitrogen). 1 ml of preculture was used to inoculate 100 ml of BMGY media containing 30 g/l nitrogen source (either ACF or animal based reference), 13.4 g/l YNB, 100 mM potassium phosphate buffer, 0.5 % methanol and 40 µg/l biotin solution. Methanol was added to a final concentration of 0.5 % every 24 hours. β-galactosidase activity was measured as per the manufacturers protocol.
Activity of extracellularly-expressed α-galactosidase:
A second protein activity assay was carried out using a *Saccharomyces cerevisiae* strain that extracellulary expresses α-galactosidase. 1 ml of preculture was added to 50 mls media containing 10 g/l nitrogen source, 13.4 g/l YNB, 21 g/l glucose and 0.2 g/l MgSo4, which was incubated at 30 C with agitation at 300 rpm.
Activity was measured by adding 225 µl fresh pNPG substrate to a test tube and placing it in a water bath at 37 C for 2 minutes. 25 ul of sample was added to the test tube and incubated for 5 mins at 37 C. 500 µl sodium carbonate was then added to the sample to develop the colour, which was measured at 405 nm..

Results

The initial step in the optimization of an ACF media formulation for recombinant protein expression in yeast was to analyze the effect of animal free peptones & yeast extracts on growth. A wide range of Kerry's ACF total nitrogen sources were tested on 3 commercially available *P. Pastoris* strains, two commercially available *H. polymorpha* strains and two of Kerry's *S.cerevisiae* strains to see which YE/peptone combinations performed well for each individual strain and across all the yeast strains tested (Figure 1).
The next step was to analyze the effect of the promising ACF formulations from the growth studies on protein expression. For the first proprietory *P. pastoris* strain, **Hy-Express™ System IV**, **Hy-Express™ System VIII** and the **Developmental Hy-Express™ 1** showed a greater amount of protein at 72 hours (Figure 2. (a)). For the second *P. pastoris* proprietary strain **Hy-Express™ System IV**, **Hy-Express™ System VI** and the **Developmental Hy-Express™ 1** & **2** show significantly more protein than the reference at 72 hours (Figure 2. (b)).
For the proprietary *S. cerevisiae* strain, **Hy-Express™ System I, Hy-Express™ System IV** & **Hy-Express™ System V** all show higher protein expression levels at 66 hours, as do **Hy-Peptone™ 1001, Hy-Peptone™ 1004 & Hy-Peptone™ 1007** (Figure 2 (c))**.** Although the majority of formulations tested exhibit improved protein yield across these strains, these SDS-PAGE gels show that in some cases the media formulation can have different effect's on protein production for different strains. No protein degradation was visible on the SDS-PAGE gels.
Lastly, a number of ACF media formulations that were promising for both growth improvement analysis and protein quality/quantity increases were tested with regard to protein activity. The β-galactosidase *P. pastoris* strain (Invitrogen) was grown in ACF media, and in animal based media as a reference. The graph represents samples taken 48hrs post induction. All the ACF systems tested are comparable to, if not greater than, the animal based references (Figure. 3 (a)). In this graph, the samples have been normalized to an animal based reference. A value of 1 corresponds to a specific activity of approximately 38,000 units/mg of protein.
Activity was also measured using a customer α-galactosidase producing *S. cerevisiae* strain.. In this this case, enzyme activity related to the Kerry ACF media is greater than the ACF reference after 64 hours (Figure 3(b)). In this graph, the samples have been normalized to an ACF reference. A value of 1 corresponds to a specific activity of approximately 16.53 units/ml.

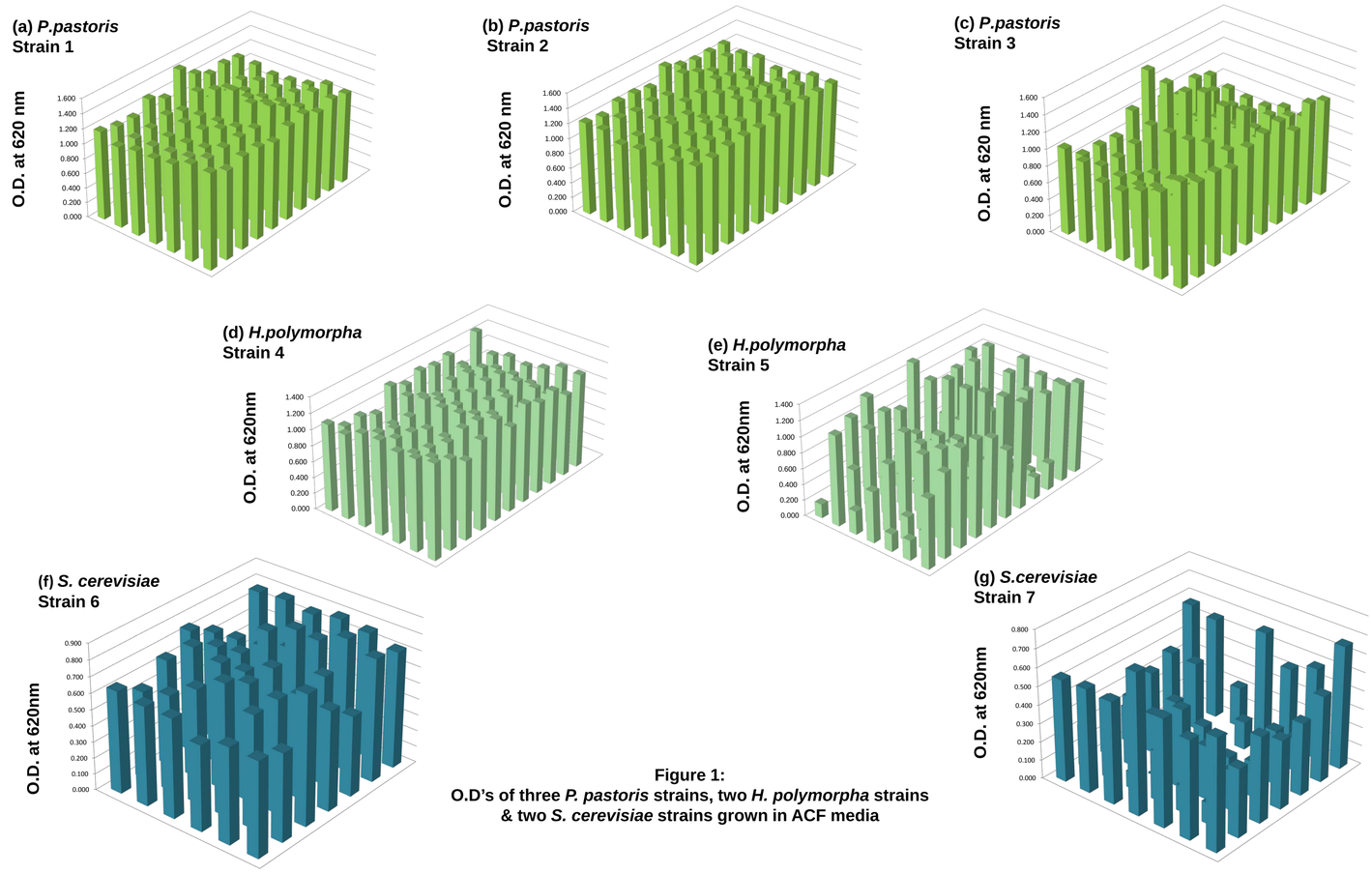

Figure 1:
O.D's of three *P. pastoris* strains, two *H. polymorpha* strains & two *S. cerevisiae* strains grown in ACF media

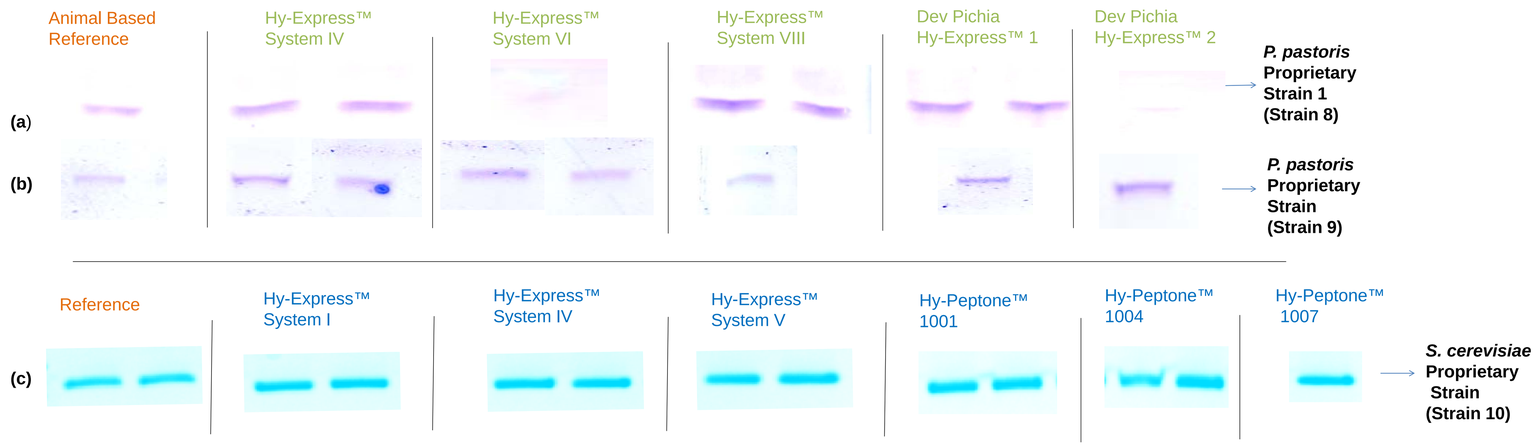

Figure 2:
(a & b) SDS Page of 2 *P. pastoris* proprietary strains comparing protein levels in animal based reference media and (c) a *S. cerevisiae* proprietary strain in a ACF reference media with Kerry ACF media.

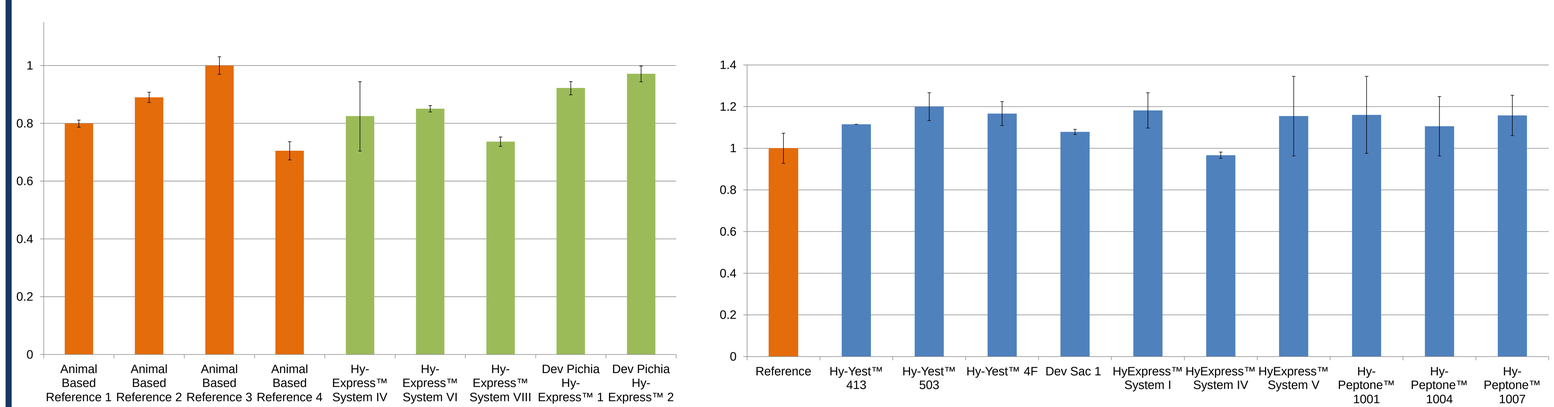

Figure 3:
(a) Normalized β-galactosidase activity results for the *Pichia Pastoris* GS115 β -gal strain (strain 11) grown in ACF media compared to a number of animal based media formulations.
(b) Normalized α-galactosidase activity results for an in house Saccharomyces Cerevisiae α -gal strain (strain 12) grown in ACF media compared to an outside/external ACF media.

Conclusion

In this study a number of Kerry ACF media formulations were analysed with regard to recombinant protein production across 11 Yeast strains. The parameters analyzed to test the effectiveness of the media were growth studies (O.D.), protein quantity/quality (SDS-PAGE) and protein activity (β-galactosidase assay & α-galactosidase assay). A number of Kerry ACF formulations performed comparable to or better than animal based formulations across all parameters tested.
With an increasing trend towards ACF media formulation, Kerry offers a number ACF media formulations that provide the benefit of maintaining or improving growth, product quantity/quality and product activity as compared with that of animal based complex media formulations. These media formulations will provide our customers with the ability to remove animal derived components from their media without adversely effecting the performance of their process.