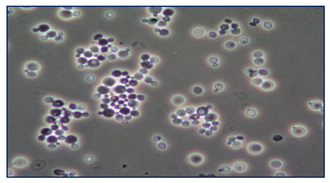


Animal-free media optimization for recombinant protein production in *Pichia pastoris*

Brian Murphy, Ignacio J. Cabrera-Ostertag and John F. Menton

Kerry, 3400 Millington Road, Beloit, WI 53511 john.menton@kerry.com - http://www.SheffieldBioScience.com

Introduction:

Pichia pastoris is a methylotrophic yeast that is quickly becoming a major platform for recombinant protein production. The use of *Pichia pastoris* has a number benefits. While possessing similar extensive growth characteristics to prokaryotic cells, they also have the ability to glycosylate and secrete recombinant proteins, a function normally associated with slower growing mammalian cells.

Pichia pastoris can be grown in both chemically defined and complex media, although growth in complex media provides a significant advantage with regard to biomass and protein production. In recent years there has been a growing trend towards the use of non-animal media components. This relates to a number of issues that have arisen including the mycoplasma contamination in the 1970's, endotoxin issues in the 80's and BSE scares in the 90's.

The purpose of this study was to optimize animal component free (ACF) media formulations for recombinant protein production in *Pichia pastoris*.

Materials and methods:

Pre-screening for growth:

Three *Pichia pastoris* strains were screened for growth response to a combination of Kerry's animal component free (ACF) protein hydrolysates and yeast extracts. The 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.

Extracellular expression of protein in *Pichia Pastoris*:

The four most promising ACF samples that boosted the performance of *P. pastoris* were reanalyzed in two proprietary *P. pastoris* strains already in use in industry. Shake flask experiments were performed where the effects on protein quantity and quality were assessed 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 *P. pastoris* 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.

Intracelullar expression of protein in *Pichia Pastoris grown in 1L fermenters*:

The Invitrogen β-galactosidase expressing strain (Pichia) was grown in BMGY fermentation media (40g/l nitrogen source, 14.82 mM potassium phosphate buffer, 13.4 g/l YNB, 40 µg/l biotin and 4 % glycerol) using the Kerry ACF nitrogen source or a Bactopeptone/Bacto YE control (animal based). The strain was grown in Sartorius 1L Biostat A Plus fermenters. The vessels were fed with air, while temperature was controlled at 30 °C and agitation set at 1000rpm. The starting pH of the fermentation was 6, while the set point was set at 5. pH was controlled using 28% NaOH. The initial growth phase on glycerol was followed by a 1 hour starvation phase. At this point methanol feeding began, where methanol was added in 6-14 g/L doses over 1 hour periods. Each methanol dose was initiated based on a fixed feeding schedule. The methanol feeding lasted 72 hours, while the total fermentation length was ca. 96 hours. Glycerol, methanol and growth were all tracked using the ASL Pichia Bioprocess Monitor.

Results

The initial step in the optimization of an ACF media formulation for recombinant protein expression in *P. pastoris* 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 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 (Figure 2). A number of peptones showed greater quantity in comparison to the reference in at least one strain if not in both. 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 (Figure 3& 4). The β-galactosidase *P. pastoris* strain (Invitrogen) was grown in ACF media, and in animal based media as a reference. Samples were taken at 24, 48 72 and 96 hours. The Kerry ACF media was comparable to if not greater than the animal based reference, with peak activity ranging from 80-120% of the reference. The ACF media also consumed on average 2.5% less methanol than the reference. Based on all the parameters tested, and the fact that we have seen some strain dependent differences, Kerry recommends **Hy-Express™ System VIII**, **Hy-Express™ System IV**, **Hy-Express™ System VI** & **Hy-Express™ System IX** for recombinant protein production in *Pichia pastoris* using animal component free media.

Conclusion

In this study a number of Kerry ACF media formulations were analyzed with regard to recombinant protein production across six *P. pastoris* 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). 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.

Acknowledgements

We would like to thank ASL Analytical ™ for providing us with the opportunity to use their Pichia Bioprocess monitor allowing us to accurately track, map and control our fermentations.

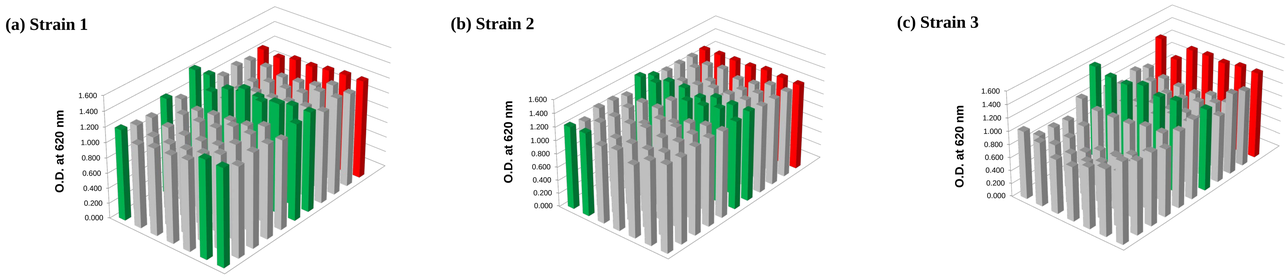


Figure 1: O.D's of three *Pichia* strains grown in ACF media and animal based media as a reference.
Strains were inoculated into 96 well plates containing a BMGY media with a range of animal free yeast extracts & peptones and animal based references. The plates were placed in a 30°C incubator at an rpm of 300 for 18hours. O.D. @ 620nm was measured using the Berthold Mithras LB940 plate reader.

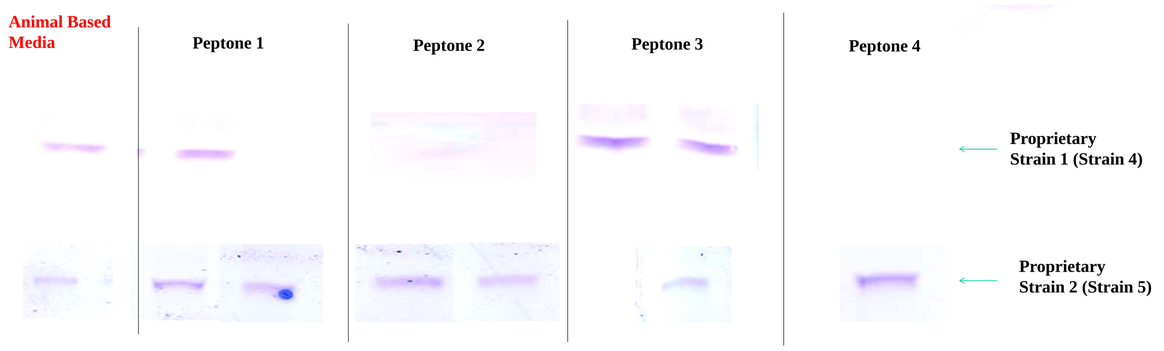


Figure 2: SDS-PAGE of two customer strains comparing protein levels in reference animal based media and Sheffield ACF media.
Strains were grown in an animal based media (already in use by the customer) and promising Kerry ACF media. Secreted target protein was analysed by harvesting the supernatant and running samples on SDS-PAGE gels, specifically looking at protein quantity and quality.

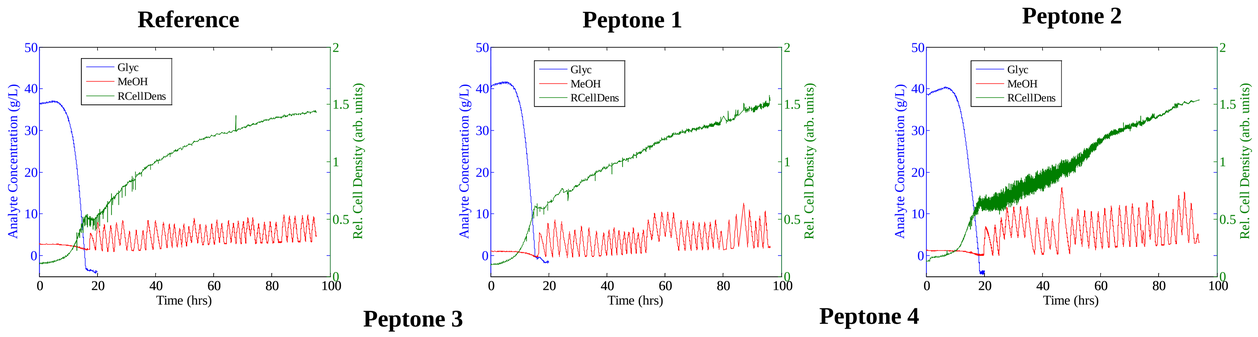
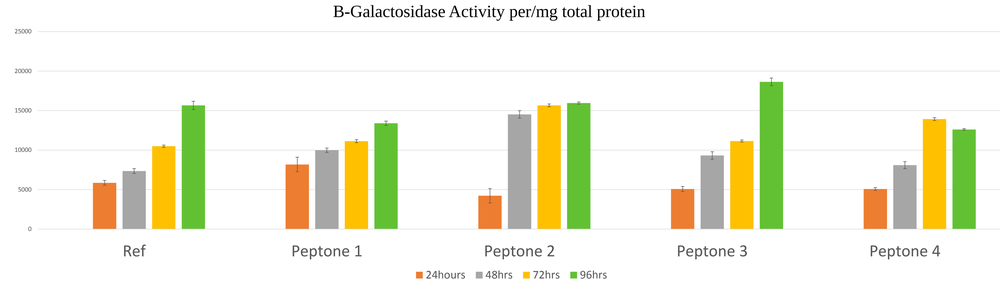


Figure 3: Glycerol, methanol and growth profiles of five Pichia fermentations-monitored using the ASL real-time bioprocess monitor.
Five half litre fermentations were carried out comparing Kerry ACF media to a bactopeptone/bacto YE control with regard to β-galactosidase activity.

(a)



(b)

Media	Peak Activity/mg total protein	Peak Time	Glycerol Consumption Rate/hr	Overall Methanol Consumption Rate/hr
Ref	15657	96	2.367g	2.00g
Peptone 1	13389	96	2.820g	1.95g
Peptone 2	15951	96	2.139g	1.69g
Peptone 3	18636	96	2.476g	1.80g
Peptone 4	13923	72	2.554g	2.35g

Figure 4: (a) β-galactosidase activity results for the *Pichia Pastoris* fermentations grown in ACF media compared to an animal based media formulation
(b) Summary of each fermentation including glycerol and methanol consumption rates