Modular development enables rapid design of media for alternative hosts

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Abstract

Developing media to sustain cell growth and production is an essential and ongoing activity in bioprocess development. Modifications to media can often address host or product-specific challenges, such as low productivity or poor product quality. For other applications, systematic design of new media can facilitate the adoption of new industrially relevant alternative hosts. Despite manifold existing methods, common approaches for optimization often remain time and labor intensive. We present here a novel approach to conventional media blending that leverages stable, simple, concentrated stock solutions to enable rapid improvement of measurable phenotypes of interest. We applied this modular methodology to generate high-performing media for two phenotypes of interest: biomass accumulation and heterologous protein production, using high-throughput, milliliter-scale batch fermentations of *Pichia pastoris* as a model system. In addition to these examples, we also created a flexible open-source package for modular blending automation on a low-cost liquid handling system to facilitate wide use of this method. Our modular blending method enables rapid, flexible media development, requiring minimal labor investment and prior knowledge of the host organism, and should enable developing improved media for other hosts and phenotypes of interest.

52 **Introduction**

53 Achieving high volumetric productivities of biologic drugs in cultivation is a key step in
54 advancing candidate biologic drugs. The outcome of this effort ultimately impacts
55 manufacturing costs as well as readiness for transitioning clinical-stage development (Love,
56 Love, & Barone, 2012). The development of standard, chemically defined media for established
57 manufacturing hosts, such as CHO, has made such transitions efficient for monoclonal
58 antibodies by achieving high biomass accumulation, cell viability, operational consistency, and
59 specific productivities, streamlining development efforts (McGillicuddy, Floris, Albrecht, &
60 Bones, 2018; Rodrigues, Costa, Henriques, Azeredo, & Oliveira, 2012). Nonetheless, optimizing
61 productivity or quality attributes for a specific product often still requires further refinement of
62 media (Ritacco, Wu, & Khetan, 2018). Such development may require evaluating dozens of
63 variants derived from a common standard formulation to address the specific challenges
64 encountered (Gagnon et al., 2011; Loebrich et al., 2019). Media development for entirely new
65 biomanufacturing technologies, such as alternative hosts (Matthews, Kuo, Love, & Love, 2017a)
66 or new product modalities (Lu et al., 2016), may also require new formulations or extensive
67 optimizations due to limited prior knowledge.

68 Common approaches to develop a medium to optimize a phenotype of interest are often
69 labor intensive, low throughput, or rely heavily on extensive analytical capacity (Galbraith,
70 Bhatia, Liu, & Yoon, 2018). For example, analysis of residual media after cultivation requires
71 extensive capabilities for analytical characterization and prior experience with the manufacturing
72 host to identify potentially limiting or toxic media components (Mohmad-Saberi et al., 2013;
73 Pereira, Kildegaard, & Andersen, 2018). As a result, optimizations can be slow and iterative.
74 Furthermore, for an alternative host such as *Komagataella phaffii* (formerly known as *Pichia*
75 *pastoris*), there is substantially less, if any, prior knowledge available to establish profiles for
76 residual components in media after fermentation. Other analytical techniques like RNA-seq
77 combined with methods for reporter metabolite analysis can guide media optimization, to

generate testable hypotheses regarding beneficial modifications to media (Matthews et al., 2017a). Such genome-scale approaches, however, require prior host-specific knowledge, such as well-annotated genomes, and are still limited by slow iteration and labor-intensive preparations of new media to test the hypotheses generated from computational analyses.

Alternative strategies for blending basal components for media allow linear combinations of existing media to explore many variations rapidly (Jordan, Voisard, Berthoud, & Tercier, 2013). While this approach avoids slow iterative analyses, the typical experiment is labor intensive to perform, often requiring independent preparations of over a dozen stock media to combine (Rouiller et al., 2013). Similar to analytical-based approaches for optimization, the selected variations of media are simultaneously guided and constrained by prior experience and media designs, which may limit the breadth of components examined (Kennedy & Krouse, 1999). For less established hosts with fewer available formulations of media, media blending may also require fully *de novo* formulations for initial studies. Further complicating such designs, different and new components for media can present challenges in solubility or unanticipated interactions with other elements in the formulations (Ritacco et al., 2018). New approaches to blending could, however, enable fast, flexible experimentation and minimize the time, labor, and analytical development needed initially to optimize media for new applications and phenotypes.

Here, we present a novel and generalizable approach for the modular development of media and demonstrate its use to create optimized media for two different phenotypes—cellular growth and recombinant expression of a protein (as measured by the secreted heterologous protein titer) from *Pichia pastoris*. Our approach comprises two modular parts for blending and optimization. We determined that a set of simple concentrated stock solutions constructed in defined modules could generate many media by blending or dilution. We then automated a simple, inexpensive liquid handling system (Opentrons OT-2) to enable high-throughput screening for the effects of diverse media on a phenotype of interest in milliliter-scale batch cultures. To maximize the benefit of this automated blending, we also developed an algorithmic

104 framework for systematic modular media optimization, beginning from a simple minimal media
105 (here a YNB-based one). This framework provides insights pertaining to key media components
106 during stages of optimization, as well as overall mapping of the design space for the media. In
107 the examples presented here, the resulting defined media developed with this strategy
108 outperformed commonly used BMGY and BMMY complex formulations for biomass
109 accumulation and secreted heterologous protein production.
110

111 **Materials and Methods**

112 *Strains and cultivation conditions*

113 Media for evaluating biomass accumulation were developed using a previously
114 described strain expressing G-CSF under control of the pAOX1 promoter (Crowell et al., 2020).
115 24-well plate screens were conducted as described previously, except cells were grown on a
116 labForce shaker and were only sampled 24 hours after inoculation (Matthews et al., 2017a).
117 BMGY, BMMY, and RDM media were formulated as described previously for shake flasks
118 (Matthews et al., 2017a). All cultivations were inoculated from a working cell bank at an initial
119 cell density of 0.1 OD/mL. For each working cell bank, cells grown in 1 L shake flasks with a 200
120 mL working volume of RDM were harvested during exponential growth (4-5.5 OD/mL) via
121 centrifugation at 1500 rcf for 4 minutes at 23 ^{o}C and resuspended in an equal volume mixture of
122 RDM and 50 v/v% glycerol. This mixture was then distributed into 700 μL aliquots and stored at
123 -80 ^{o}C, resulting in a cell density of ~30 OD/mL for the cell bank.

124 Media for evaluating enhanced production were developed using a strain expressing a
125 rotavirus-derived subunit vaccine candidate, P[8], under the control of the pAOX1 promoter
126 described previously (Dalvie et al., 2020). Biomass accumulation proceeded for 24 hours; cells
127 reached an initial induction density of ~20 OD/mL. Cultures were then exchanged into
128 production media and allowed to produce protein for an additional 24 hours. Supernatant was
129 harvested by centrifugation at 1500 rcf for 4 minutes at 23 ^{o}C and filtered using a Captiva 96

130 well 0.2 μm filter plate (Agilent Technologies, Santa Clara, CA) prior to titer measurement by
131 RP-UHPLC.
132 Media components and supplements were purchased from Sigma-Aldrich, St. Louis,
133 MO, unless otherwise indicated in the supporting information. A table of supplement and stock
134 solutions with screening concentrations is also included in the supporting information. During
135 modular optimization, all media were prepared in high throughput using an Opentrons OT-2
136 liquid handler (Opentrons, Brooklyn, NY, software version ≥3.16.1) using Openblend. Modular
137 media blending code and instructions for setup and operation are provided in the Openblend
138 package (https://github.com/abiedermann/openblend_public). For consistency, media used in
139 final head-to-head comparisons were prepared in bulk and filter sterilized through a 0.2 μm
140 benchtop filter.
141
142 *Analytical procedures*
143 Biomass was measured by optical density at 600 nm as described previously (Matthews
144 et al., 2017a). An Agilent Bravo liquid handler was used to dilute samples prior to
145 measurements of OD into the Tecan Infinite M200 Pro plate reader.
146 Reverse phase ultra-high performance liquid chromatography (UHPLC) analysis was
147 performed on Agilent 1290 Infinity II UHPLC system controlled using OpenLab CDS software
148 (Agilent Technologies, Santa Clara, CA). The concentration of protein was determined using a
149 Poroshell 120 SB-Aq column (2.1 x 50 mm, 1.9μm) operated at 1.0 mL/min and 70 ^{o}C (Agilent
150 Technologies, Santa Clara, CA). Buffer A was 0.1% (v/v) TFA in water and buffer B was 0.1%
151 (v/v) TFA, 0.5% (v/v) water in ACN. A gradient was performed as follows: 30% B for 1 min., 30-
152 40% B over 3 min., 40-90% B over 0.5 min., 90% B for 0.5 min., 90-30%B over 0.5 min., and
153 30% for 1 min.; total method run time was 6.5 minutes. Sample injection volumes were 50μL. A
154 diode array detector was set for absorbance detection at 214nm. Data analysis was completed
155 using OpenLab CDS Data Analysis (Agilent Technologies, Santa Clara, CA).

156 Statistical analysis and DOE design was conducted using JMP (SAS Institute, Cary NC).
157 Quadratic models were fitted using effect screening and non-significant terms (adjusted p-value
158 > 0.01) were eliminated sequentially in order of decreasing adjusted p-value to avoid overfitting.
159 Data was plotted using Prism 8.4.0 (GraphPad Software, San Diego, CA).
160
161
162
163 **Results**
164 *Design of approach for modular media blending*
165 We sought to develop an approach capable of identifying important, beneficial
166 modifications for media tailored to a given phenotype of interest. We reasoned that key
167 requirements for such an approach would be that it is fast and automatable, with minimal
168 dependence on complex analytical assay development. Such features would enable routine
169 application to any measurable phenotype of interest. In general, media blending allows both
170 speed and low analytical complexity. We aimed to retain these features while minimizing the
171 labor and constraints on compositions imposed by linear combinations of fully formed and
172 unique media. We reasoned that diverse and flexible blends of media could be created by
173 defining simple concentrated stock solutions as basic modules to combine further. These
174 modules would comprise individual components or common subsets of components with
175 compatible solubilities (e.g. YNB). If media components could be formulated in concentrated
176 stock solutions that could be stored stably over time, then the components could be routinely
177 and interchangeably combined and diluted to the desired final concentrations. This approach
178 would yield a broadly applicable modular strategy for media blending amenable to conventional
179 liquid handling automation.
180 To test the feasibility of this approach, we first assessed whether many common media
181 components could be formulated in concentrated stable aqueous stock solutions. Using the

182 CHO medium eRDF as a reference, we estimated the solubility of each component of this
183 medium, using data from AqSolDB as well as other online sources (Combs, 2012; FSA Panel
184 on Additives and Products or Substances used in Animal Feed (FEEDAP), 2011; Ritacco et al.,
185 2018; Schnellbaecher, Binder, Bellmaine, & Zimmer, 2019; Sorkun, Khetan, & Er, 2019;
186 Yamamoto & Ishihara, n.d.). We compared the estimated solubility of each media component to
187 its concentration in eRDF and found that, individually, most media components are soluble at
188 levels >10x higher than their eRDF concentration (**Figure 1A**). The existence of a wide range of
189 commercially available concentrated supplements further supports this result: >50x
190 concentrated solutions of amino acids, vitamins, lipids, and trace metal supplements are
191 common and commercially available.

192 Next, we used the product information of commercially available supplements, literature
193 sources, and inspection to estimate the percentage of eRDF media components that could be
194 stored in stable solutions for >6 months. We estimated that over 75% of eRDF components met
195 this criterion (**Figure 1B**). To address stability challenges caused by less stable components,
196 we reasoned that less stable components or supplements, such as vitamins, could be prepared,
197 aliquoted, and stored frozen for long-term storage (Schnellbaecher et al., 2019); these aliquots
198 could then be thawed and used within a defined period to mitigate component instability and
199 enable their integration into our modular blending strategy. Together, these solubility and
200 stability data suggested that a modular approach to media development could be defined in this
201 way to accommodate a range of new formulations easily.

202 We next automated the process for constructing media, using the Opentrons OT-2. We
203 chose this liquid handler due to its low cost, reliability, and compatibility with simple formats for
204 data input, such as Excel spreadsheets. We then created an open-source Python package,
205 named Openblend, which simplified the media construction process by handling routine
206 experimental design and execution steps (Figure 1C). Openblend creates an experimental
207 design spreadsheet, specifying the number of 24 well plates, the desired media composition of

208 each well, and stock solution names and concentrations. The script then checks the feasibility
209 of the experimental design, ensuring that the total volume of each well will not exceed the target
210 volume and avoiding the addition of sub-microliter stock solution volumes. If the design passes
211 this assessment, the script then outputs a new spreadsheet containing the setup for the OT-2
212 deck and required volumes of stock solutions, providing a user with instructions on how to setup
213 the OT-2 liquid handler. We found that our typical time to execute this script, setup the OT-2
214 and initiate plate building was ~15 minutes, and the time for the automated steps was about two
215 hours.

216 Finally, we defined a modular approach for optimization to effectively leverage the
217 Openblend tool (**Figure 1D**). Beginning from an initial basal medium, improved media are
218 constructed through successive rounds of optimization. In each round, a library of media
219 components and supplements are screened to identify beneficial additives. These additives are
220 then screened in combination and over a range of concentrations to further optimize the
221 performance of the medium. Each modular addition and optimization of additives can be guided
222 simply by measurements of the phenotype of interest (e.g. biomass accumulation). This greedy
223 approach to multi-dimensional optimization could continue iteratively until the resulting media
224 met desired specifications, all available media components were explored, or no additional
225 gains in performance realized.

226

227 *Application to Developing a Medium for Biomass Accumulation*

228 To assess the utility of this blending-based approach, we next aimed to identify and
229 optimize the concentration of media components beneficial for rapid biomass accumulation of *P.*
230 *pastoris* in batch cultivation. We previously described a rich defined medium (RDM) (Matthews
231 et al., 2017a), capable of high growth rates during biomass accumulation. One challenge
232 encountered with this formulation, however, was that precipitates can form at higher pH values
233 that require filtering during bulk preparations. Nonetheless, this medium provided a relevant

234 comparison for assessing the medium realized with our new approach due to its prior
235 demonstrated benefits relative to complex media. Following our modular approach, we
236 improved biomass accumulation by optimizing the accumulated optical density at 600 nm after
237 24 hours of cultivation.

238 Algorithms for optimizing systems based on multiple dimensions are often sensitive to
239 initial conditions used (Zakharova & Minashina, 2015). Given this potential confounding effect
240 here, we tested first the effects of the types of carbon source, nitrogen source, and pH set point
241 on biomass accumulation, using 1x YNB without amino acids or ammonium sulfate (YNB) to
242 satisfy minimum requirements for the concentrations of trace elements. We conducted a full-
243 factorial DOE using glycerol, glucose, and fructose as carbon sources; urea and ammonium
244 sulfate as nitrogen sources; and potassium phosphate as a buffer with pH values of 5, 5.75, and
245 6.5. We selected initial concentrations of 40 g/L, 4 g/L urea or the N-mol equivalent for
246 ammonium sulfate, and 10 g/L potassium phosphate, similar to values used in other media for
247 *Pichia pastoris* (Matthews et al., 2017a). A least squares regression model, including individual,
248 combination, and quadratic effects was fit to the log of optical density after 24 hours, a proxy
249 variable for the average growth rate (R^2 = 0.81). We determined that the two most significant
250 model terms were the type of carbon source and the interaction of the nitrogen source with pH
251 (**Figure 2A**). We found that cells grew significantly faster on metabolically related sugars
252 (glucose and fructose) than on the polyol (glycerol) commonly used for Pichia during biomass
253 accumulation (**Figure 2B**). This result affirms prior reports where glucose has been used for
254 biomass accumulation of Pichia (Guo et al., 2012; Moser et al., 2017).

255 The model also suggested that poor biomass accumulation during cultivation resulted
256 from a combination of ammonium sulfate as a source of nitrogen with low buffer pH (**Figure**
257 **2B**). This outcome may result from the production of acidic species associated with cellular
258 ammonium metabolism in the batch cultivation (Villadsen, 2015). Interestingly, the model
259 indicated slightly greater biomass was achieved with urea instead of ammonium sulfate. The

260 biomass accumulation of cultures grown with urea as a source of nitrogen were less sensitive to
261 reduced pH values (~5). We observed, however, that cultivations at pH 5 showed extensive
262 flocculation compared to those at 6.5. Given the insensitivity of urea-fed cultivations to buffer
263 pH and the high solubility and potential for low-cost sourcing of fructose, we therefore chose to
264 include fructose, urea, and a potassium phosphate buffer with a pH of 6.5 in our initial media
265 formulation.

266 With this basal formulation determined, we next screened for concentration-dependent
267 interactions of other key additives to the media and then optimized concentration-dependent
268 parameters. Following the same approach for screening effects, we conducted a full factorial
269 DOE over a broad range of media component concentrations: YNB (0.5, 1, 2x), fructose (10, 30,
270 50 g/L), urea (1, 4, 7 g/L), and potassium phosphate adjusted to a pH of 6.5 (4, 10, 16 g/L). The
271 resulting model identified fructose as a concentration-sensitive parameter (R^2=0.73) (**Figure**
272 **2D**). Terms involving the concentration of YNB were also highly ranked, but not statistically
273 significant. No significant interactions between components were identified in the model. We
274 therefore sought to better understand the concentration dependence of fructose and YNB
275 independently (**Figure 2E**), over an 8-fold range of concentrations. As expected, biomass
276 accumulation was highly sensitive to fructose concentration, with an optimum around 22.5 g/L of
277 fructose. The concentration of YNB had minimal effect on biomass accumulation; the presence
278 of trace elements supplied by YNB, however, was essential to growth. Based on these results,
279 we chose concentrations of 22.5 g/L fructose, 1x YNB, 7 g/L urea, and 10 g/L potassium
280 phosphate buffer. We reasoned that although biomass accumulation was relatively insensitive
281 to the concentrations of YNB and urea, higher concentrations could provide improved media
282 depth in future applications. We named this basal formulation DM1_dev0.

283 We next assessed what additional media components could improve biomass
284 accumulation. To test over 60 different components individually would require over 60 individual
285 solutions. Such an approach would scale linearly with new components; instead, we chose to

286 screen groups of related components, using commercially available pre-mixed supplements. We
287 compiled a library of 16 commercial supplements and industrially-relevant surfactants containing
288 more than 60 unique components and screened their individual effect on biomass accumulation
289 after 24 hours. In this way, we reasoned we could efficiently identify critical classes of
290 components related to the phenotype of interest and potentially deconvolve specific individual
291 additives of interest by inference. We used the recommended concentrations of each
292 supplement as supplied in product information, or critical micelle concentrations, and prior
293 knowledge for broad classes in yeast media to set reasonable screening concentrations
294 (Supporting Information). We identified five beneficial and two detrimental supplements that
295 significantly impacted biomass accumulation ($p_{adj} < 0.02$; 1-way-ANOVA) (**Figure 2F**). In
296 general, the results suggest that supplementation with amino acids and trace metals were
297 beneficial for accumulating biomass, while two surfactants, Tween 20 and CHAPS, were
298 detrimental. For this phenotype, the effects of vitamin and lipid supplements were minor;
299 supplements from either supplement category were not significantly beneficial or detrimental to
300 biomass accumulation. Our earlier experiments suggest that vitamins are essential but
301 concentration agnostic (**Figure 1E**), while lipid supplementation provides no clear benefit for
302 biomass accumulation.

303 Based on these results, we chose to test whether combinations of supplements of amino
304 acids and trace salts could yield synergistic improvements in biomass accumulation. We
305 screened pairwise combinations of the five beneficial supplements of mixed composition and
306 ranked the performance of our supplementation strategies (**Figure 2G**). A combination of 1x
307 MEM amino acids with 0.1 v/v% PTM1 salts resulted in the highest yield of biomass, though we
308 observed strong performance from other combinations of amino acid and trace metal
309 supplements. Based on these data, we chose to add MEM amino acids and PTM1 salts in our
310 basal medium and optimized their concentrations (**Figure 2H**).

311 Based on these results, we elected 0.1 v/v% PTM1 salts and 1x MEM amino acids, in
312 order to balance the moderate benefits and potentially high costs of amino acids. We found,
313 however, that the inclusion of the PTM1 salts in liter-scale preparations produced fine
314 precipitates, which can impede sterile transfers in use. To overcome this challenge, we
315 screened a broad range of PTM1 salts concentrations to identify the minimum concentration
316 required for improved outgrowth performance (**Figure 2I**). We found that PTM1 addition at
317 concentrations as low as 0.0005 v/v% led to increased biomass accumulation. We therefore
318 revised our PTM1 salts concentration to 0.01 v/v%, a concentration high enough to obtain the
319 benefits of PTM1 supplementation without inducing precipitate formation. This formulation we
320 named DM1.
321 Completing this series of optimizations with our iterative modular approach to define a
322 new formulation of medium, we then compared with other common media used to grow *P.*
323 *pastoris*. We evaluated the performance of this new optimized medium (DM1) relative to the
324 unsupplemented basal medium (DM1_dev0), the rich defined medium (RDM) we had previously
325 developed, and a common medium 4 v/v% glycerol BMGY. We found that DM1 yielded the
326 highest biomass accumulation, with significantly higher biomass accumulation relative to RDM
327 and BMGY (**Figure 2J**). This result demonstrates the utility of our modular strategy here for
328 media development that yielded an improved formulation for biomass accumulation compared
329 to other common media with minimal time and labor investment, and without requiring complex
330 analytical methods like mass spectrometry or RNA-sequencing.
331
332 *Identifying media conditions important to heterologous protein production in* K. phaffii
333 In addition to the time and labor savings of modular media development, our proof-of-
334 concept experiments demonstrated that this approach creates a flexible medium that can be
335 rapidly adapted to new growth phenotypes, as well as a data package that the identifies media
336 conditions important to the phenotype of interest. We reasoned that these additional benefits

337 could be particularly relevant for optimizing production of heterologous proteins. Understanding
338 which media components contribute most significantly to productivity could improve culture
339 performance and help identify important metabolic pathways or physiological effects for further
340 study.

341 To develop a medium for improved production of a recombinant protein, we chose to use
342 a strain engineered to secrete a rotavirus-derived subunit vaccine component, VP4-P[8], as a
343 model protein. We have previously demonstrated that this viral antigen can be expressed at
344 high titer under the control of the methanol-inducible pAOX1 promoter in BMMY media (Dalvie
345 et al., 2020). Similar to our initial approach to optimize a medium for growing biomass, we first
346 determined and optimized the concentrations of the sources for carbon and nitrogen, along with
347 the pH. The expression of P[8] in the strain tested uses the methanol-dependent pAOX1
348 promoter for inducible expression, so we selected methanol as the initial carbon source. We
349 then examined the impact of the source of nitrogen and buffer pH on titer. We conducted a full-
350 factorial DOE using identical concentrations as those used to create a medium for accumulating
351 biomass. The resulting model was visualized by ranking combinations of sources of nitrogen
352 and buffer (**Figure 3A**). The effects showed no interaction between these two factors. Urea was
353 again identified as the preferred source of nitrogen while higher pH values led to improved
354 secreted P[8] productivity. Unlike biomass accumulation, this pH dependence was observed
355 across both nitrogen sources.

356 We next applied the same DOE to identify important concentration-dependent
357 interactions that impact the production of P[8]. Unsurprisingly, the concentration of methanol
358 was the most important factor, with possible minor effects from other components (**Figure 3B**).
359 We decided to screen further a 20-fold range in methanol concentrations using two formulations
360 for remaining media components—the one determined for optimal cell growth (DM1) and the
361 optimal base media formulation predicted by the quadratic model here (2x YNB, 1 g/L urea, 4
362 g/L potassium phosphate adjusted to a pH of 6.5). We found that production was relatively

363 insensitive for concentrations of methanol ranging from 1-4 v/v%, with an optimum around 2%
364 (**Figure 3C**). We postulated that the rapid decline in productivity observed in these milliliter-
365 scale cultures using concentrations >6 v/v% methanol was likely due to excess formation of
366 toxic metabolic byproducts such as formaldehyde and hydrogen peroxide (Wakayama et al.,
367 2016). Interestingly, the predicted optimal medium from this set of studies outperformed the
368 medium we determined for accumulating biomass, suggesting that certain components of the
369 basal medium may benefit protein expression more than cellular growth and underscores the
370 value of optimizing media for specific phenotypes of interest. Based on these data in total, we
371 defined a basal medium for production including 2x YNB, 2 v/v% methanol, 1 g/L urea, and 4
372 g/L potassium phosphate buffer adjusted to a pH of 6.5 (DM2_dev0).
373 Next, we examined which supplements could improve the performance of DM2_dev0.
374 We added three chemical chaperones (TUDCA, sodium deoxycholate monohydrate (SDM), and
375 valproic acid) (Kuryatov, Mukherjee, & Lindstrom, 2013; Uppala, Gani, & Ramaiah, 2017), two
376 antioxidants (reduced glutathione (GSH) and N-acetyl cysteine (NAC)), and the chelator, K-
377 ETDA, to the list of 16 supplements included in our original screen defined for biomass
378 accumulation. Concentrations for these components were chosen based on product
379 specifications, literature data, and prior experience (Supporting Information). Many of the 22
380 supplements screened improved production of P[8] (**Figure 3D**). The top four ranking
381 supplements comprised surfactants or lipids, which could modulate membrane fluidity and lipid
382 metabolism (Butler, Huzel, Barnab, Gray, & Bajno, 1999; Degreif, Cucu, Budin, Thiel, & Bertl,
383 2019; Ritacco, Frank V; Yongqi Wu, 2018).
384 We then screened combinations of lipid supplements and surfactants to identify potential
385 synergistic effects. We ranked the individual supplements and their combinations (**Figure 3E**)
386 according to the measured titers of P[8]. We found that the addition of a cholesterol-rich
387 supplement yielded the highest secreted titers of P[8] (~50% improvement compared with
388 supplement-free condition in initial screens). Interestingly, a synthetic cholesterol supplement

389 alone did not substantially improve performance, suggesting the benefit results from a
390 combination of fatty acids and surfactant components in the supplement (**Supporting**
391 **Information**). This conclusion is consistent with similar improvements observed from other
392 supplements, such as linoleic acid-oleic acid-albumin (**Figure 3D**).

393 Since no other synergistic effects were observed in the combination screen, we
394 assessed the dependence of titer on the concentration of the cholesterol-containing supplement
395 identified (**Figure 3F**). Similar to our observations with cellular YNB used in the outgrowth
396 media, we found that concentrations of the supplement as low as 0.2 v/v% were beneficial for
397 protein expression, but that production was relatively insensitive to concentration (**Figures 3F,**
398 **3G**). We then directly compared the supplemented medium to the original composition; the new
399 supplemented media provided a 25% improvement in titer ($p = 0.0006$, one-tailed Welch's T
400 test). This new formulation with 1x cholesterol supplement, which we named DM2_dev1, was
401 the result of one cycle of optimization using our method.

402 Components of the cholesterol supplement included fatty acids, cholesterol, and
403 cyclodextrin, which are all are known to modulate membrane fluidity, a key parameter in vesicle
404 trafficking (Cooper, 1978; Degreif et al., 2019; Mahammad & Parmryd, 2015). We reasoned that
405 the addition of this supplement could therefore have synergistic effects with other supplements,
406 but did not find any further supplementation that improved P[8] titers within our original screen
407 (**Figure 3H**). We, therefore, considered if there could be additional classes of beneficial
408 supplements, absent from the original screen. Previous experiments demonstrated that P[8]
409 productivity is highly sensitive to methanol concentration (**Figure 3C**), so we wondered whether
410 further modulation of central carbon metabolism could yield additional productivity gains.

411 Modification of central carbon metabolism is best accomplished by feeding cells
412 alternative carbon sources, either entirely or as co-feeding substrates. Four co-fed substrates
413 have previously been shown to be non-repressive of pAOX1: sorbitol, mannitol, trehalose, and
414 alanine (Inan & Meagher, 2001). These substrates can be co-utilized with methanol without

415 repressing the pAOX1 promoter, which controls expression of P[8]. We hypothesized that the
416 introduction of supplemental carbon sources could enable further optimization of central carbon
417 metabolism. We screened co-fed substrates individually and in 1:1 combinations at a total
418 concentration of 20 g/L (a concentration similar to the optimal fructose and methanol
419 concentrations observed in previous carbon source optimizations) (**Figure 2E,3C**). Sorbitol co-
420 feeding had the most beneficial effect, resulting in a ~80% increase in P[8] titer (**Figure 3I**).
421 Mannitol supplementation was also beneficial (~70% increase), while alanine and trehalose co-
422 feeding were detrimental to productivity. While co-feeding carbon sources led to increased
423 biomass yield during production, these differences did not account for the improved titer, as
424 improvements in specific productivity (q_p) of ~60% and ~45% were also observed for the sorbitol
425 and mannitol co-fed conditions, respectively (**Supporting Information**). Based on these data,
426 we chose to include sorbitol as a supplemental carbon source for further study.
427 The addition of a supplemental carbon source could significantly impact central carbon
428 metabolism. We, therefore, wondered how the inclusion of sorbitol might impact the optimal
429 carbon feeding strategy. Examining total carbon source concentrations from 20 – 70 g/L, we
430 compared the performance of cultures co-fed with sorbitol:methanol ratios of 3:1, 1:1, and 1:3 to
431 a methanol-only control (**Figure 3J**). All co-fed conditions outperformed the methanol-only
432 control, suggesting that the presence of sorbitol is highly beneficial for producing P[8]. The titer
433 was relatively insensitive to sorbitol:methanol ratios and carbon concentrations. Based on the
434 data, we decided to use 2 v/v% methanol and 20 g/L of sorbitol for the final sorbitol-
435 supplemented media named DM2.
436 Finally, we compared the P[8] titer obtained using DM2_dev0, DM2_dev1, and DM2 to
437 other common production media for *P. pastoris*: BMMY and RDM. We found that DM2 led to a
438 ~2x improvement in P[8] titers, relative to BMMY and RDM, up to 97 $\pm$ 2 mg/L.
439
440 **Discussion**

441 Here we have implemented a novel and broadly applicable approach for media
442 development that relies on rapid, automated construction of diverse media from defined
443 modules of components. We demonstrated the utility of this approach by developing two new
444 media for two phenotypes of interest in the heterologous production of proteins by yeast,
445 namely biomass accumulation and secreted production. We systematically identified and
446 optimized the concentration of media components important to each phenotype of interest.
447 Importantly, defining these new formulations of media did not require advanced analytical
448 capabilities and required minimal experimental time to assess more than 360 total formulations
449 during two to three rounds of optimization for each.
450 Our optimized formulations affirmed the importance of lipid-related components for
451 maximizing titers in *Pichia pastoris* cultivations. The importance of optimizing membrane fluidity
452 or lipid metabolism has been well established in CHO and appears to be key to optimizing
453 heterologous protein secretion in *P. pastoris* cultivation as well (Clincke et al., n.d.; Ritacco et
454 al., 2018; Zhang, Wang, & Liu, 2013).
455 Modular media blending has four advantages over existing methods. First, the use of
456 common stock solutions and supplements to formulate media reduces initial labor required for
457 new experiments or optimizations ~15 minutes per experiment, making parallel testing of
458 multiple hypotheses efficient and requires less resources overall. Here, we created 30 stock
459 solutions, and evaluated >360 unique media compositions, without manual preparation of
460 individual media or extensive blending calculations or planning. Most of these solutions could be
461 readily reused in future experiments to optimize for new phenotypes of interest. Second, our
462 method requires minimal knowledge of the host organism *a priori* and could, in principle, be
463 applied to any measurable phenotype of interest. We anticipate that this method could be used
464 to optimize other phenotypes of interest, such as glycosylation profiles. Third, our method
465 provides certain practical advantages, including minimal requirements for analytical
466 characterization and rapid identification of component interactions that lead to solubility

467 challenges. These traits make it possible to learn about formulations that may lead to extensive
468 precipitates like those encountered with our rich defined medium formulation (**Figure 4A**).
469 Finally, modularly constructed media, such as DM2, can be ~70% pure water with low
470 osmolarity, leaving volumetric and osmotic space for future modifications to accommodate new
471 or related phenotypes of interest (**Figure 4B**).

472 We also acknowledge certain limitations in the present study that may be addressed in
473 future work. First, while modular media development identifies components key to the
474 optimization of the phenotype of interest, additional media optimization effort may be necessary
475 to translate these learning in batch cultivations to scaled-up fed-batch or perfusion operation,
476 where additional variables such as supplemental feed composition and feeding schedule must
477 also be considered. In principle, modular media construction could be applied to high-
478 throughput scale-down cultivation models, such as Ambr250s. Second, our approach for
479 optimization relies on greedy algorithms tailored to create a new media for a single phenotype of
480 interest; however, given the vast explorable media space it is possible to find a local optimum.
481 Further metabolic or -omic modeling techniques could be employed to guide broader exploration
482 of media space, co-optimize multiple phenotypes, or facilitate biologically informed optimization,
483 albeit with more complex experimental and computational requirements (Matthews, Kuo, Love,
484 & Love, 2017b; Mohmad-Saberi et al., 2013). Third, our current method used commercially
485 available supplements, but in practice, beneficial supplements could be simplified by using
486 individual components, to facilitate more biological inferences and aid development of improved
487 host-specific supplements. Finally, initial screens to identify beneficial supplements rely on
488 reasonable choices of initial concentrations for screening. These currently require prior
489 knowledge from the literature or commercial sources; with further use in the community of the
490 Openblend approach, it is possible additional sharing of knowledge could help inform further
491 developments.

The improved speed and accessibility of in-depth media development experiments enabled by modular media construction could help improve expression of many classes of proteins in laboratories and discovery centers that have not traditionally had access to such capabilities. Since many lead candidates for new therapeutic proteins begin in small biotech firms and academic labs, early-stage improvements in productivity could help advance more proteins towards the clinic simply by facilitating access to larger quantities of proteins for initial research and non-clinical studies. In more established companies, the ability to make rapid improvements to existing media may enable faster product development timelines and could reduce manufacturing costs overall. Rapid identification and optimization of sensitive media components could also enable easier adoption of a range of industrially relevant alternative hosts, resulting in further manufacturing flexibility and potentially cost savings (Coleman, 2020).

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A.M.B., I.R.G., K.R.L., and J.C.L. conceived and planned experiments. A.M.B. conducted media development experiments. S.R.A. developed and maintained the RP-UPLC assay. A.M.B. performed analytical characterization. A.M.B., K.R.L., and J.C.L. wrote the manuscript. J.C.L. and K.R.L. designed the experimental strategy and supervised analysis. All authors reviewed the manuscript.

516 **References**
517

518 Butler, M., Huzel, N., Barnab, N., Gray, T., & Bajno, L. (1999). Linoleic acid improves the
519 robustness of cells in agitated cultures, 27–36.
520 Clincke, M.-F., Guedon, E., Yen, F. T., Ea, L., Universite, N., Ogier, V., … Goergen, J.-L. (n.d.).
521 Effect of Surfactant Pluronic F-68 on CHO Cell Growth , Metabolism , Production , and
522 Glycosylation of Human Recombinant IFN- c in Mild Operating Conditions, 181–190.
523 https://doi.org/10.1002/btpr.503
524 Coleman, E. (2020). Establishment of a Novel Pichia Pastoris Host Production Platform by.
525 Combs, G. F. (2012). *The vitamins: Fundamental aspects in nutrition and health*. San Diego:
526 Elsevier Academic Press.
527 Cooper, R. A. (1978). Influence of Increased Membrane Cholesterol on Membrane Fluidity and
528 Cell Function in Human Red Blood Cells. *Journal of Supramolecular Structure*.
529 Crowell, L. E., Crowell, L. E., Raymond, A., St, H. E., Engineering, C., Doyle, P. S., & Crowell,
530 L. E. (2020). Accelerating process development for biologics on an automated , pharmacy-
531 scale manufacturing system by by.
532 Dalvie, N. C., Brady, J. R., Crowell, L. E., Tracey, M. K., Biedermann, A. M., Kaur, K., … Love,
533 J. C. (2020). Molecular engineering improves antigen quality and enables integrated
534 manufacturing of a trivalent subunit vaccine candidate for rotavirus. *BioRx*, 1–51.
535 Degreif, D., Cucu, B., Budin, I., Thiel, G., & Bertl, A. (2019). Lipid determinants of endocytosis
536 and exocytosis in budding yeast. *BBA - Molecular and Cell Biology of Lipids*, *1864*(7),
537 1005–1016. https://doi.org/10.1016/j.bbalip.2019.03.007
538 FSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2011).
539 Scientific Opinion on safety and efficacy of choline chloride as a feed additive for all animal
540 species. *EFSA Journal*, *9*(9), 2353.
541 Gagnon, M., Hiller, G., Luan, Y. T., Kittredge, A., Defelice, J., & Drapeau, D. (2011). High-End
542 pH-controlled delivery of glucose effectively suppresses lactate accumulation in CHO Fed-
543 batch cultures. *Biotechnology and Bioengineering*, *108*(6), 1328–1337.
544 https://doi.org/10.1002/bit.23072
545 Galbraith, S. C., Bhatia, H., Liu, H., & Yoon, S. (2018). Media formulation optimization: current
546 and future opportunities. *Current Opinion in Chemical Engineering*, *22*, 42–47.
547 https://doi.org/10.1016/j.coche.2018.08.004
548 Guo, C., Huang, Y., Zheng, H., Tang, L., He, J., Xiang, L., … Jiang, H. (2012). Secretion and
549 activity of antimicrobial peptide cecropin D expressed in Pichia pastoris. *Experimental and*
550 *Therapeutic Medicine*, 1063–1068. https://doi.org/10.3892/etm.2012.719
551 Inan, M., & Meagher, M. M. (2001). Non-repressing carbon sources for alcohol oxidase (AOX1)
552 Promoter of Pichia pastoris, *92*(6), 585–589.
553 Jordan, M., Voisard, D., Berthoud, A., & Tercier, L. (2013). Cell culture medium improvement by
554 rigorous shuffling of components using media blending, 31–40.
555 https://doi.org/10.1007/s10616-012-9462-1
556 Kennedy, M., & Krouse, D. (1999). Strategies for improving fermentation medium performance:
557 a review. *Journal of Industrial Microbiology and Biotechnology*, (23), 456–475.
558 Kuryatov, A., Mukherjee, J., & Lindstrom, J. (2013). Chemical Chaperones Exceed the
559 Chaperone Effects of RIC-3 in Promoting Assembly of Functional a 7 AChRs. *PLoS ONE*,
560 *8*(4), 1–11. https://doi.org/10.1371/journal.pone.0062246
561 Loebrich, S., Clark, E., Ladd, K., Takahashi, S., Brousseau, A., Kitchener, S., … Ryll, T. (2019).
562 Comprehensive manipulation of glycosylation profiles across development scales. *MAbs*,
563 *11*(2), 335–349. https://doi.org/10.1080/19420862.2018.1527665
564 Love, J. C., Love, K. R., & Barone, P. W. (2012). Enabling global access to high-quality
565 biopharmaceuticals. *Current Opinion in Chemical Engineering*, *2*(4), 383–390.
566 https://doi.org/10.1016/j.coche.2013.09.002

Lu, T. L., Pugach, O., Somerville, R., Rosenberg, S. A., Kochenderfer, J. N., Better, M., & Feldman, S. A. (2016). A Rapid Cell Expansion Process for Production of Engineered Autologous CAR-T Cell Therapies. *Human Gene Therapy Methods*, *27*(6), 209–219. https://doi.org/10.1089/hgtb.2016.120

Mahammad, S., & Parmryd, I. (2015). Cholesterol depletion using methyl-β-cyclodextrin. In *Methods in membrane lipids* (pp. 91–102).

Matthews, C. B., Kuo, A., Love, K. R., & Love, J. C. (2017a). Development of a general defined medium for Pichia pastoris, (July), 103–113. https://doi.org/10.1002/bit.26440

Matthews, C. B., Kuo, A., Love, K. R., & Love, J. C. (2017b). Development of a general defined medium for Pichia pastoris, (July), 103–113. https://doi.org/10.1002/bit.26440

McGillicuddy, N., Floris, P., Albrecht, S., & Bones, J. (2018). Examining the sources of variability in cell culture media used for biopharmaceutical production. *Biotechnology Letters*, *40*(1), 5–21. https://doi.org/10.1007/s10529-017-2437-8

Mohmad-Saberi, S. E., Hashim, Y. Z. H. Y., Mel, M., Amid, A., Ahmad-Raus, R., & Packeer-Mohamed, V. (2013). Metabolomics profiling of extracellular metabolites in CHO-K1 cells cultured in different types of growth media. *Cytotechnology*, *65*(4), 577–586. https://doi.org/10.1007/s10616-012-9508-4

Moser, J. W., Prielhofer, R., Gerner, S. M., Graf, A. B., Wilson, I. B. H., Mattanovich, D., & Dragosits, M. (2017). Implications of evolutionary engineering for growth and recombinant protein production in methanol - based growth media in the yeast Pichia pastoris. *Microbial Cell Factories*, 1–16. https://doi.org/10.1186/s12934-017-0661-5

Pereira, S., Kildegaard, H. F., & Andersen, M. R. (2018). Impact of CHO Metabolism on Cell Growth and Protein Production: An Overview of Toxic and Inhibiting Metabolites and Nutrients. *Biotechnology Journal*, *13*(3), 1–13. https://doi.org/10.1002/biot.201700499

Ritacco, Frank V; Yongqi Wu, A. K. (2018). Cell Culture Media for Recombinant Protein Expression in Chinese Hamster Ovary (CHO) Cells : History , Key Components , and Optimization Strategies. https://doi.org/10.1002/btpr.2706

Ritacco, F. V., Wu, Y., & Khetan, A. (2018). Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. *Biotechnology Progress*, *34*(6), 1407–1426. https://doi.org/10.1002/btpr.2706

Rodrigues, M. E., Costa, A. R., Henriques, M., Azeredo, J., & Oliveira, R. (2012). Comparison of commercial serum-free media for CHO-K1 cell growth and monoclonal antibody production. *International Journal of Pharmaceutics*, *437*(1–2), 303–305. https://doi.org/10.1016/j.ijpharm.2012.08.002

Rouiller, Y., Périlleux, A., Collet, N., Jordan, M., Stettler, M., & Broly, H. (2013). A high-throughput media design approach for high performance mammalian fed-batch cultures, (June), 501–511.

Schnellbaecher, A., Binder, D., Bellmaine, S., & Zimmer, A. (2019). Vitamins in cell culture media: Stability and stabilization strategies. *Biotechnology and Bioengineering*, *116*(6), 1537–1555. https://doi.org/10.1002/bit.26942

Sorkun, M. C., Khetan, A., & Er, S. (2019). AqSolDB , a curated reference set of aqueous solubility and 2D descriptors for a diverse set of compounds, *2019*(July), 1–8. https://doi.org/10.1038/s41597-019-0151-1

Uppala, J. K., Gani, A. R., & Ramaiah, K. V. A. (2017). Chemical chaperone , TUDCA unlike PBA , mitigates protein aggregation efficiently and resists ER and non-ER stress induced HepG2 cell death. *Scientific*, *1*(January), 1–13. https://doi.org/10.1038/s41598-017-03940-1

Villadsen, J. (2015). Redox Balances and Consistency Check of Experiments. In *Fundamental Bioengineering* (pp. 17–38).

Wakayama, K., Yamaguchi, S., Takeuchi, A., Mizumura, T., Ozawa, S., Tomizuka, N., …

Nakagawa, T. (2016). Regulation of intracellular formaldehyde toxicity during methanol metabolism of the methylotrophic yeast Pichia methanolica. *Journal of Bioscience and Bioengineering*, *122*(5), 545–549. https://doi.org/10.1016/j.jbiosc.2016.03.022

Yamamoto, T., & Ishihara, K. (n.d.). Stability of Glutathione in Solution. *Developments in Food Engineering*, 209–211.

Zakharova, E. M., & Minashina, I. K. (2015). Review of Multidimensional Optimization Methods. *Mathematical Models, Computational Methods*, *60*(6), 625–636. https://doi.org/10.1134/S1064226915060194

Zhang, H., Wang, H., & Liu, M. (2013). Rational development of a serum-free medium and fed-batch process for a GS-CHO cell line expressing recombinant antibody, 363–378. https://doi.org/10.1007/s10616-012-9488-4

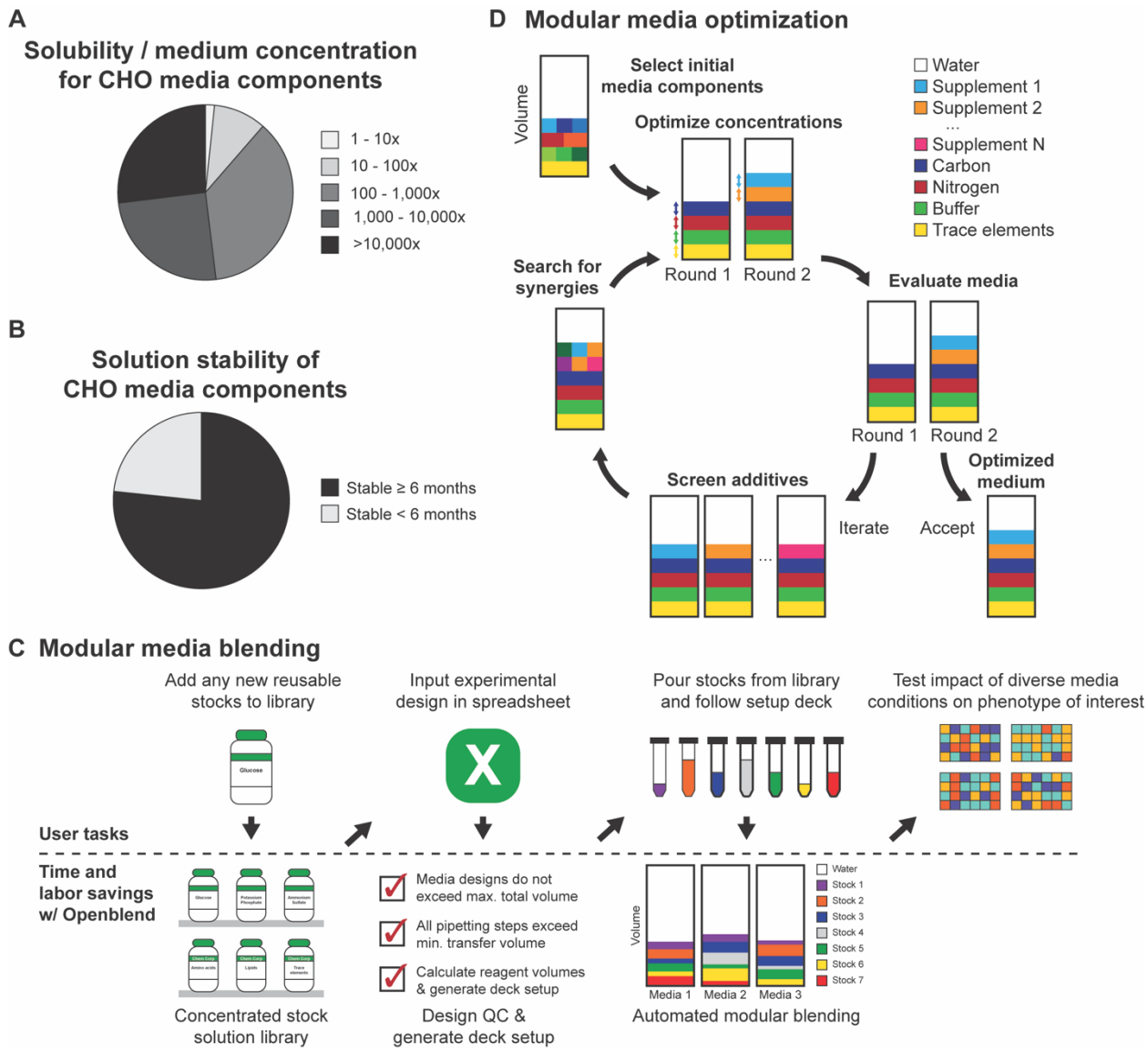

Figure 1. Modular media development can be broadly applicable, easily applied, and systematically executed to improve measurable phenotypes of interest.

A) Estimate of the ratios of component solubility to their concentrations in medium demonstrates that most components are soluble at >10x their concentration in the CHO medium, eRDF. B) With the exception of some classes of medium components, such as vitamins, most media components can be formulated into solutions that remain stable for >6 months under proper storage conditions. C) Overview of time, labor and planning saved by using Openblend to automate modular media construction. D) Overview of a modular media optimization approach,

640 which can be used to build an optimized medium for any measurable phenotype of interest

641 systematically.

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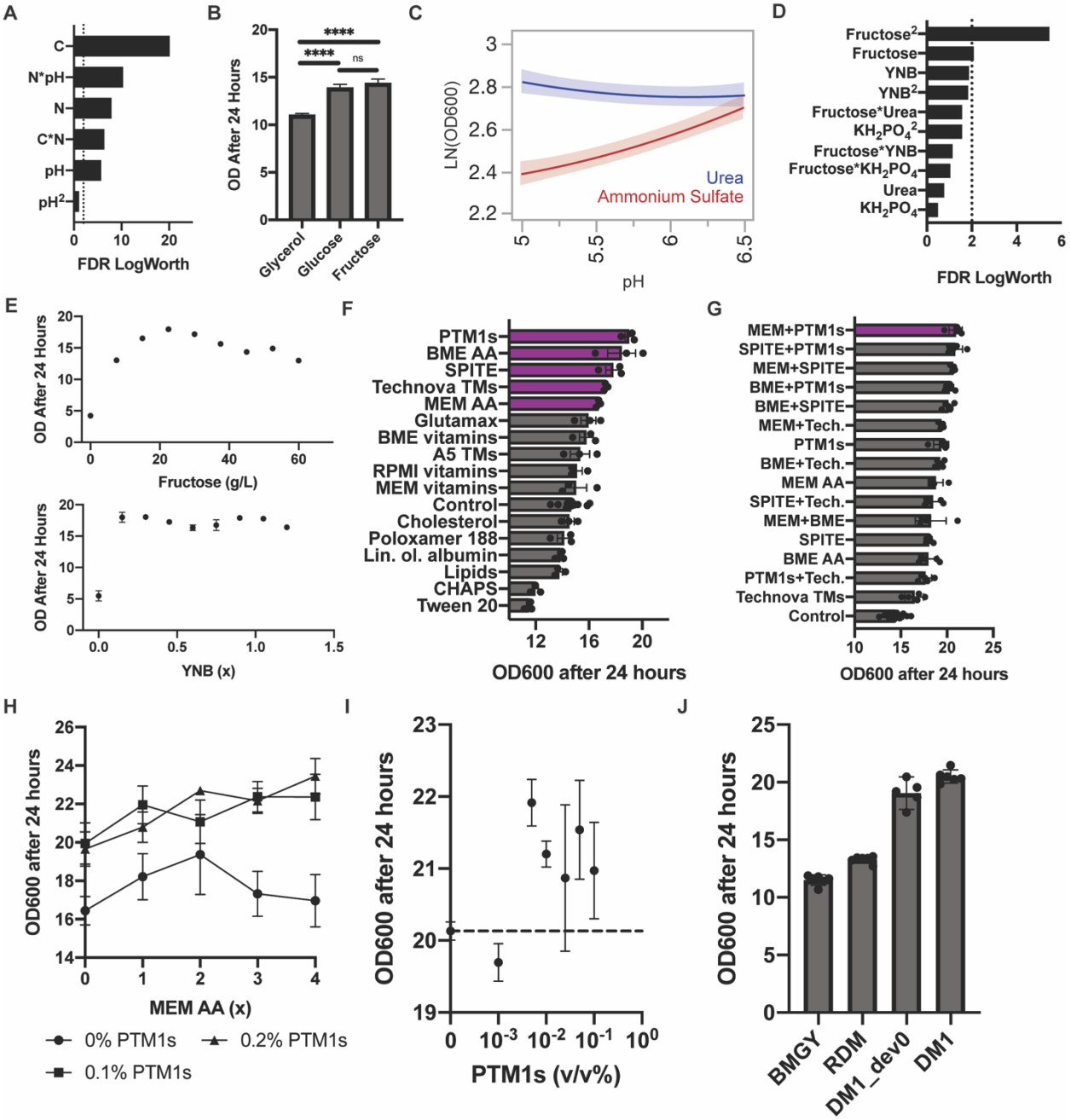

643

644 **Figure 2**. Modular development of a new biomass accumulation media for *P. pastoris*

645 A) Significance of carbon (fructose, glucose, glycerol), nitrogen (urea and ammonium sulfate),

646 and pH choice (5, 5.75, 6.5) in a least square regression model fitted to a full factorial DOE. B)

647 Fructose and glucose were found to result in significantly higher biomass accumulation after 24

hours of outgrowth than glycerol. C) Ammonium sulfate was found to be more pH sensitive than urea, as shown by the JMP sensitivity profiles during fructose feeding. D) Significance of terms in a least square regression model fitted to a full factorial DOE over fructose, urea, potassium phosphate, and YNB concentrations. E) 1-FAAT optimization of fructose and YNB concentration finds optimal outgrowth performance at a fructose concentration of 22.5 g/L and relative insensitivity over a wide range of YNB concentrations (0.15 to 1.2x). F) A media supplementation screen identified 5 beneficial supplements, related to trace element and amino acid supplementation. G) Further screening of beneficial supplement combinations identified synergistic amino acid and trace metal supplementation strategies. H) Comparison of the effect of MEM amino acid concentration on biomass accumulation at different PTM1 salts concentrations. I) Effect of the concentration of PTM1 salts on biomass accumulation in DM1_dev0 medium supplemented with 1x MEM AA. J) Head-to-head comparison of 4 v/v% glycerol BMGY, 4 v/v% glycerol rich defined medium, the initial defined biomass accumulation media (DM1_dev0), and the final biomass accumulation medium obtained after a full optimization cycle (DM1), demonstrates that DM1 leads to superior biomass accumulation.

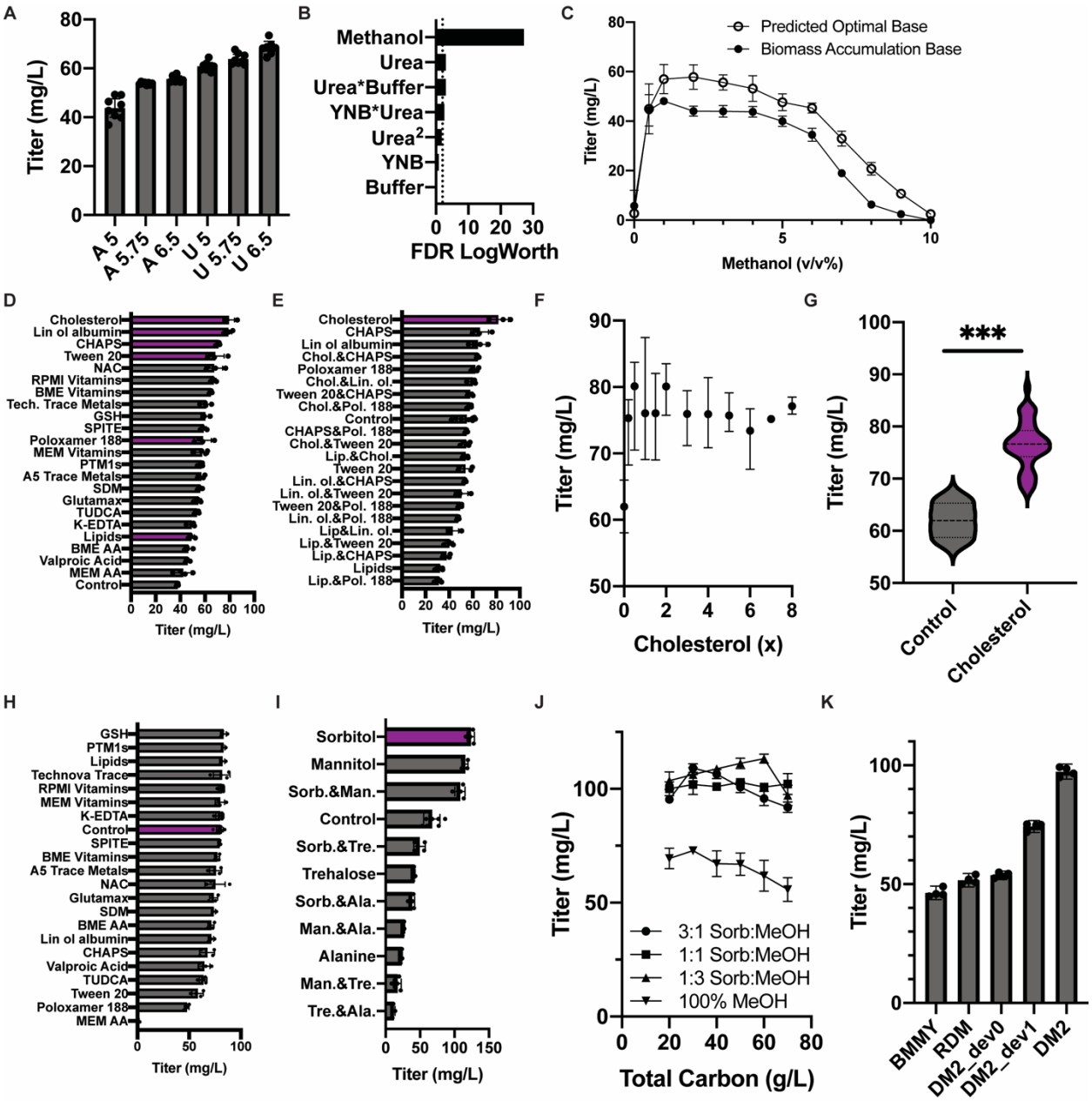

Figure 3. Modular development of a media for heterologous protein production in *P. pastoris* A) Initial full-factorial screen of nitrogen source choice and buffer pH demonstrates that urea is preferred over ammonium sulfates and high buffer pH is preferred over lower values. B) A full-factorial concentration optimization identified methanol as the most concentration dependent variable. Other components in the base media were predicted to affect productivity with much lower levels of significance. C) Evaluation of the effect of methanol concentration on P[8] titer, using two different base media (urea, buffer, and YNB concentrations): the biomass

accumulation base medium and the optimal base media composition predicted by our concentration DOE. D) Ranking of supplements according to their effect on P[8] titer. Supplements related to membrane fluidity or lipid metabolism ranked highly. E) Evaluation of combinations of lipid and surfactant supplements confirmed that cholesterol supplementation leads to the greatest improvement in P[8] titer. F) Concentration optimization of cholesterol demonstrated low concentration dependence, with similar performance observed over a 40-fold range (0.2-8x). G) Comparing cholesterol-free and cholesterol-supplemented cultures fed at various concentrations demonstrates that cholesterol supplementation results in a significant ~25% improvement in P[8] titers ($p<0.001$). H) No significantly beneficial supplements were observed when repeating the supplementation screen. I) Screening supplementation of 20 g/L of co-fed substrates individually or in 1:1 combinations by mass identified sorbitol supplementation as highly beneficial to P[8] titer. J) Examination of the effect of co-feed ratio and total carbon concentration on titer in DM2_dev1 supplemented media. K) Comparison of P[8] titer obtained with DM2 to previous iterations and other common *P. pastoris* media demonstrates a ~2x improvement in P[8] titer, relative to 1 v/v% methanol RDM and 1 v/v% methanol BMMY.

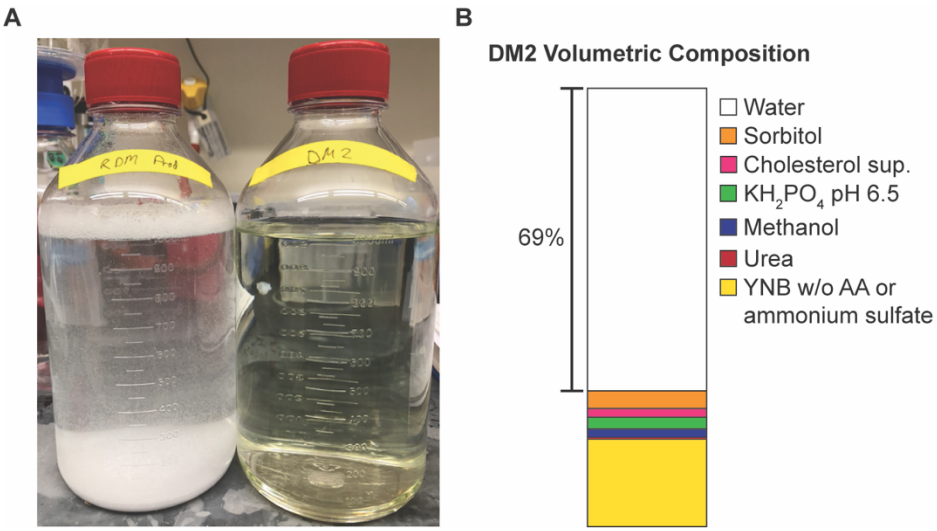

Figure 4. Comparison of DM2 to rich define medium.

A) Comparison of precipitate formation during construction of RDM (left) and DM2 (right) media. Adjusting the pH of RDM to 6.5 results in significant formation of white precipitate. No precipitate formation is observed in DM2. B) Relative volumes of stock solutions and pure water needed to construct DM2. Pure water addition accounts for 69% of DM2 volume, demonstrating that there is substantial room for further supplement exploration and development. When separated into simple stock solutions, DM2 can be 3x concentrated.