Development and Optimisation of a Defined High Cell Density Yeast Medium

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1 Abstract

The growth rate and cell density of *Saccharomyces cerevisiae* cells grown in defined media is insufficient for assays that require a sufficient cell number in a small volume. To ameliorate this problem, we developed and optimised a new high-cell density (HCD) for *S. cerevisiae*. Starting from defined media conditions, we varied the concentration of glucose, yeast nitrogen base, amino acids, mono-sodium gluatamate and inositol, and used response surface methodology (RSM) to develop and optimise the media culture conditions. We monitored growth, cell number and cell size to ensure that the optimisation was towards a greater density of cells rather than towards larger cells. Cells exhibit growth similar to the complex media YPD in our final HCD medium, while the final pre-diauxic shift cell density increased about threefold. We found normal cell-cycle behaviour throughout the growth phases by monitoring DNA content and protein expression using fluorescent reporters. We also ensured that HCD media could be used with a variety of strains and that HCD media could be used to select for all common yeast auxotrophic markers.

Keywords

S. cerevisiae, Growth Media, Growth Medium, Optimisation, Response surface methodology, Assay miniaturization, cell to cell variability, protein secretion, protein production

2 Introduction

The yeast *Saccharomyces cerevisiae* is a broadly used eukaryotic model organism in basic research as well as an important tool in biotechnology [1]. In contrast to many other model organisms, yeasts offer comparatively simple genetic manipulation using either plasmid-based expression or genome modifications [11]. Over the last 70 years, the community established a series of commonly used selection markers for this purpose, but their use requires a defined growth media for auxotrophic selection [12]. Most commonly, a defined growth media uses Glucose as its only carbon source with concentrations varying between 10 g/ℓ up to 60 g/ℓ. Additionally, a nitrogen source—in most cases ammonium sulfate—and phosphate, sulfate, and a combination of vitamins are required. The latter components all come from yeast nitrogen base (YNB), which might need to be supplemented with inositol for balanced growth of certain lab strains. Lastly, the most common defined media have a set of amino acids (aa) added, whose composition and concentrations vary among recipes and laboratory [14]. While these media have a clearly defined composition, the cell density at the diauxic shift is lower and the growth rate of *S. cerevisiae* slower compared to cells grown in the complex media YPD.

The majority of work with *S. cerevisiae* is performed on agar plates when scoring colony growth or in shaking cultures of volumes between 5 and 500 mℓ medium especially when performing biochemical assays. Increasing the throughput of agar work can be easily achieved using arrayed colonies on standard SPS format plates [7]. Scale-up of the throughput of liquid experiments, however, critically depends on the sensitivity of the readout and ease of cell lysis [18]. Prime examples for successful miniaturisation are transcriptional reporter assays where a readout of interest can be induced and read by sensitive methods such as β-galactosidase, or scoring of single cell fluorescence using flow cytometry [21, 20]. Meanwhile, miniaturisation of classical biochemical assays is more daunting. For example, already 1.7 mℓ of culture needed to be grown in the complex media YPD for a simple study of the level of expressed protein using Western blotting with the sensitive TAP tag [10]. Even larger volumes are required for co-immunoprecipitation using the TAP tag or any other tag [23], except when the analysis is performed using sensitive, state-or-the-art mass spectrometry [9, 8]. For the vast majority of labs, miniaturisation of co-immunoprecipitation is therefore unattainable, especially when proteins are expressed at low levels.

A crucial step towards miniaturisation of all biochemical assays is therefore the increase in the amount of starting material by increasing the cell density at the diauxic shift. It is conceivable, that this can be achieved by optimisation of the concentration of the ingredients of the commonly used growth medium, while keeping the growth rate similar to not dramatically alter the cellular physiology. Obtaining such a media would allow more labs to perform biochemical assays using standard SPS-plates and aids in lifting the readout above the detection limit.

Optimising growth conditions is a standard procedure to make biotechnolog-

ical production processes economically viable, mainly as more cells per volume require less production capacity, generate less waste, and give more yield per volume [27]. However, most of these optimisation start from a complex media and then add buffer substances, carbon sources and vitamins to obtain a high cell density [19, 26, 15]. These media do not allow for the use of auxotrophic markers and are therefore ill-suited for basic research. Only few media composition allow for balanced growth during the exponential phase and increased secretion of proteins, but do not increase the cell density [29, 13].

A well-established statistical technique for iterative optimisation is the *Response surface method (RSM)*. Here, one uses the amounts of a medium's k components as explanatory variables $x_1, \ldots, x_n$ and interprets a defined response $y(x_1, \ldots, x_k)$ as a function of these variables (the *response surface*) [2, 3]. One then aims a determining the optimum of this response surface via sequential experimentation, iterating between a step for (locally) exploring the shape of the response surface, followed by determining the direction of steepest ascent and experimentally determining y along this gradient.

Here, we started from a defined medium composition and considered altering $k = 5$ components with concentrations $x_1, \ldots, x_5$. For exploring the response surface around a given composition, one defines three sets of experimentally conditions that—taken together—allow a relatively accurate approximation of the response surface. First, one considers each component at a lower and a higher value from its current condition. All these values together define the corners of a cube. After shifting and re-scaling the coordinate system, one can consider the starting condition as the *center point* at the origin $(0, 0, 0, 0, 0)$ and the $2^5 = 32$ corners as *factorial points* with coordinates $(\pm 1, \pm 1, \pm 1, \pm 1, \pm 1)$. Additionally, one determines the $2 \times 5 = 10$ *axial points* with coordinates $(\pm 1, 0, 0, 0, 0)$, $(0, \pm 1, 0, 0, 0))$ and so on. Together, these form a total of $1 + 32 + 10 = 43$ *design points* for which the response value y is determined experimentally. This number can be substantially reduced when the full factorial on $2^5 = 32$ points is replaced by a half-fraction design using $2^{5-1} = 16$ points. It is also often advantageous to provide multiple measurements of the center point, for example $n = 6$ replicates to estimate the variability, leading to a total of 48 experimental conditions, which can be reduced to 32 by using a half-fraction. This allows one to determine the residual standard deviation directly from replicates and to separate residual error from goodness-of-fit error in the statistical analysis. One then estimates the response surface around the current condition by a quadratic regression model of the form

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_5 x_5 + \beta_{12} x_x x_2 + \cdots + \beta_{45} x_4 x_5 + \beta_{11} x_1^2 + \cdots + \beta_{55} x_5^2 + e \ ,$$

where $e \sim N(0, \sigma^2)$ is the residual error. This model has one intercept β_0 (corresponding to the average response at the center point), five linear parameters β_i, five quadratic parameters β_{ii} and ten mixed parameters β_{ij}, a total of 21 parameters, which can be estimated, e.g., using least-squares. It provides a local approximation of the response surface around the current condition and accounts for proportional changes in the response as well as curvature.

In the second step, one determines the gradient of steepest ascent from the fitted model. This gives a direction along which the five ingredients are increased in fixed proportions, and which provides the steepest increase in the response y. One can then follow this gradient experimentally by measuring the response at distances, e.g., $0.0, 0.5, \ldots, 5.0$ from the starting condition along the gradient, where distances above roughly 1.5 correspond to conditions outside the range of the previous exploratory step. The highest measured response along the gradient of steepest ascent can then serve as a new central point and a new response surface can be described and a gradient of steepest ascent calculated.

Here we present an optimised synthetic well-defined media allowing for growth of *S. cerevisiae* lab strains to high cell densities and usable with common auxotrophic or drug resistancy markers. We term it *high cell density (HCD)* media. The HCD media shows tenfold more cells at the diauxic shift compared to synthetic minimal media and threefold to YPD. Importantly, it shows balanced growth during 12h of cultivation as scored by growth rate and cell-to-cell variability of growth rate sensitive reporter genes. The media was obtained by using two rounds of surface response methodology based designs. Importantly, we only optimise the concentrations of the ingredients of the starting media, a slightly adapted version of the commonly used synthetic minimal media. During the optimisation, the media was not only optimised for more Glucose, but lead us to dramatically increase the amount of amino acids and YNB, while keeping the nitrogen source mono sodium glutamate almost unchanged. We confirmed that the media is broadly applicable, using a series of auxotrophic markers as well as lab strains. The HCD media can be used to amend and miniaturise biochemical assays, such that they can be performed in 96 deep well plates. The media is favourable for any protein production related test cases using lab strains, as it leads to an increased and constant per cell secretion of the test protein amylase during the whole growth phase.

3 Results

3.1 Optimisation using experimental design

In order to apply the response surface method, we need to specify a central point and a response to be optimised. We decided to optimise growth of the widely used BY strain background, using a mat a strain, bearing his3Δ, leu2Δ, ura3Δ, met15Δ, and lys2Δ gene deletions. We then chose our central point based on a YNB supplemented version of a previously published optimised yeast media to optimally support growth of the BY strain series [14]. A simple growth test revealed that this starting media already outperforms the standard media. To simplify the experimental design, we created 5 blocks: Glucose concentration (C-source), mono-Sodium Glutamate as nitrogen source (N1, we wanted to keep the media compatible with drug resistancy markers), the mix of aa supplementing growth as well as additional aa required for fast growth of the BY strains (N2), YNB (Vit1), and inositol (Vit2).

Table 1: Design points for first RSM exploration around initial medium composition.

Component	**Design points**		
	0	−1	+1
Glucose	4%	2%	6%
MSG (N1)	2mg/mℓ	1mg/mℓ	3mg/mℓ
AA pool (N2)	1×	0.5×	2×
YNB (Vit1)	3mg/mℓ	2mg/mℓ	4mg/mℓ
Inositol (Vit2)	1mg/mℓ	0mg/mℓ	2mg/mℓ

Since we are interested in optimising the number of cells, we measured the growth curve for each design point using a Biolector growth reader, and used the increase in optical density ΔOD (Figure 1A) from the start of growth to the beginning of the diauxic shift as our response function y that we aimed to maximise. This gives a crude but sufficient approximation of the increase in cell number. We also need to specify how much each ingredient should be altered in the exploration step, i.e., what concentrations or amounts the coordinates ± 1 refer to, and these values are given in Table 1 for the first iteration.

After performing the two steps outlined above, we found that the predicted growth response versus the measured growth response matched well for the first six points along the gradient of steepest ascent (Figure 1B), and we took the sixth point at distance 2.5 from the starting medium as our new optimum composition. This intermediate optimum corresponded to a composition of 3% glucose, 2.7 mg/mℓ MSG (N1), 3.3× amino acid pool (N2), 10.8 mg/mℓ YNB (Vit1), 1.4 mg/mℓ inositol (Vit2).

In order to ensure that the direction of the ascent was indeed towards more cells and not simply towards more biomass, we checked if the cells behaved normally with regards to cell size, we measured cell size at the end of growth in the Biolector using a Coulter counter. We found no indication of abnormal cell sizes and also did not find any obvious correlation between cell size profile and a specific media component, suggesting that it is indeed a combination of several components that affects cell size distribution.

Using the intermediate optimum composition as our new center point, we iterated the two steps of response surface exploration and gradient-pursuit again and arrived at a second, increased, intermediate response for a medium composition of 8.1% glucose, 5.2 mg/mℓ MSG (N1), 6.9× amino acid pool (N2), 2.7 mg/mℓ YNB (Vit1), 1.9 mg/mℓ inositol (Vit2). To ensure reproducibility of our results, we additionally performed two independent replicates of the exploration and subsequent gradient pursuit experiments. Both replicates are in excellent agreement, as are the predicted and experimental responses (Figure 2A). The vertical shift between predictions and measurements is likely due to slight differences in calibration of the growth reader between the first and second round of optimisation, which we performed several weeks apart.

In contrast to the first iteration, however, we found that higher responses

were often associated with substantially slower growth and with increasing cell size, suggesting that this second iteration of optimisation was more focused towards increasing total cell mass rather than total cell number. In order to see if even more could be gained in terms of cell growth, we therefore repeated the calculation of the gradient of steepest ascent from the data of the second iteration, using an adapted response function $y = \Delta\text{OD} - 2 \cdot \Delta t$ to also include the time Δt elapsed between initiation of growth and reaching the diauxic shift (Figure 1A). This new response function again favours large increases in optical density, but additionally penalises long time intervals.

We again checked the prediction such that no conditions in the vicinity showed a decreased max growth or growth rate or increased cell size. Our data shows a clear optimum in growth, suggesting that we could further increase the amount of cells without decreasing the growth rate or increasing the cell size and—importantly—while keeping the cell cycle distribution constant. Similar to the second gradient, we observed that the predicted responses along the new gradient were overly optimistic. Nevertheless, the measured responses followed a clear upward trend, indicating that the corresponding medium composition lead indeed to higher cell numbers (Figure 2B).

The final medium composition from this experiment achieves cells numbers of 1.7×10^8 cells/mℓ in comparison with 1.6×10^7 cells/mℓ for SD and 6.0×10^7 cells/mℓ YPD in the exponential phase of growth. We named this new medium *HCD* for *high-cell density* medium with glucose as the carbon source. Its composition is shown in Table 2. In Figure 3, we compare typical growth curves for relevant media.

Table 2: HCD Media Composition

Component	**Final Concentration**
Glucose	6 %
MSG	4.5 mg/mℓ
YNB	12 mg/mℓ
Inositol	1.8 μg/mℓ
His	210 μg/mℓ
Leu	660 μg/mℓ
Lys	720 μg/mℓ
Met	240 μg/mℓ
Phe	300 μg/mℓ
Ser	225 μg/mℓ
Thr	120 μg/mℓ
Ura	240 μg/mℓ

Having achieved a defined media that appears to outperform SD medium in terms of growth rate and cell density, we wanted to ensure that the HCD medium behaved like a classical defined yeast medium. In particular, we wanted to ensure that the cell-cycle behaviour, the ability to maintain plasmids, and the

ability to support growth of a variety of strains remained similar to SD media.

3.2 Cells grown in HCD medium exhibit normal cell cycle behaviour

To examine that cells grown in HCD medium exhibited normal cell cycle behaviour we first looked at DNA content in logarithmically growing cultures by staining the DNA with Sytox Green (Figure 4A). We found that cultures grown in HCD medium exhibited a similar ratios of cells in G1 and G2/M as SD and YPD, with a similar number of cells in S-phase as YPD. Indicating that cells grown in HCD behave similarly to cells grown in YPD.

We further evaluated the cell cycle behaviour by examining the amount of protein expressed in the different growth phases. To examine this, we used a set of well-characterised, constitutive promoters; P_{TDH3}, P_{TEF2}, P_{ADH1}, or P_{ACT1}, driving the expression of the fluorescent reporter, citrine [17] (Figure 4B). We scored the respective expression levels at different growth phases in HCD, YPD, and SD media using flow cytometry. We found that the expression in HCD is similar to that of YPD, except for P_{TEF2} which shows a remarkable bimodal expression peak past the inflection point of the growth curve. Interestingly, all promoters show the tendency of bimodal expression in SD medium. Taken together, HCD medium showed the lowest variability for all promoters and all growth phases, followed by YPD, and SD showed a dramatic increase.

3.3 HCD medium is versatile as it can be used with common auxotrophic markers as well as different yeast species

It is important that a medium is not specific for a single strain background. As such, we used a set of common lab strain backgrounds to test their behaviour in HCD in comparison to YPD and SD. We tested CEN.PK, W303-1B, and the wild isolate RM11 [5]. For W303-1B and CEN.PK it was necessary to supplement HCD with 10× adenine and/or 10× tryptophan. Like we observed for BY4741, all strains exhibited similar growth to YPD, with extended growth phase and reaching higher ODs pre-diauxic shift (Figure 5A).

We next wanted to examine HCD media lacking specific amino acids, as auxotrophic markers are essential tools in yeast research that vary among strain backgrounds, are used to maintain plasmids and to confirm genomic integrations. As such, it is important that they can still be used with the HCD medium. To test this we grew the prototrophic strain, FY4, in HCD lacking one amino acid supplement at a time (HCD-aa) or lacking amino acids corresponding to all common auxotrophic markers (HCD-HLUMK) in the Biolector (Figure 5B). With the exception of medium lacking methionine, the HCD dropout media showed only minimal deviation from the fully supplemented growth.

3.4 HCD medium can be used for miniaturisation of assays

One of the motivations for designing a defined high cell density medium was to facilitate the miniaturisation of assays such that assays classically performed in flasks could be performed in a more efficient manner using multi-well plates. As a proof of concept, we measured the secretion of α-amylase from BY4741 cells harbouring a plasmid expressing α-amylase with a synthetic secretion signal [24] (Figure 6). We chose α-amylase as the is likely to be a hard to secrete protein as it is relatively larger, has an odd number of cysteines and is glycosylated. Expression of this protein leads to a considerable amount of oxidative stress [24].

We grew cells in multi-well deep well plates in 600 $\mu\ell$ of HCD or SD medium lacking uracil. We harvested at different growth phases and examined amylase activity in the supernatant by monitoring the release of p-nitrophenol (pNP) caused by amylase cleavage of ethylidene-pNP-G7. We were not able to detect any α-amylase activity at any stage of growth in SD medium. In contrast, we observed significant activity in the supernatants of cultures in stationary phase and late-log phase of cells grown in HCD medium. As expected, we observed more amylase secretion in growing cells compared with those in stationary phase.

4 Discussion

Miniaturising biochemical assays in *S. cerevisiae* is hard, as the commonly used strains, plasmids and auxotrophic markers only grow in a media with a short exponential phase reaching small cell numbers at the diauxic shift. Using RSM, we obtained a variant of the commonly used synthetic media with an altered composition of its ingredients. We termed the media HCD, for high cell density. Culture grown in HCD media reached an at least three- to tenfold higher density at the diauxic shift than YPD or SD, respectively, and the balanced growth phase lasted up to to 10h compared to 3-4h for the two standard media. The HCD media is compatible with widely distributed lab strains, as well as commonly used auxotrophic marker genes and combinations thereof. This is illustrated by the fact, that the auxotrophic strain (BY4741) grows similarly in fully supplemented HCD as the prototrophic strain (FY4). The media also supports positive selection as shown using the kanMX marker gene. The physiology of the cells growing in HCD seems normal, as their size remains similar to cells grown in standard conditions and the cell cycle distribution lays between cells grown in YPD an SD. Importantly, the cell to cell variability of fluorescent proteins expressed from standard promoters is constant throughout the balanced growth phase. Additionally, the cell to cell variability is smaller than both SD and YPD grown cells, indicating that all cells experience a similar environment throughout the cultivation. Taken together, the results consistently showed that the HCD media outperforms not only SD, but also YPD in terms of length of a balanced growth phase, cell numbers reached at the diauxic shift, and uniform protein expression, all while growing at a rate closer to YPD than SD.

Response surface methodology is widely used in process optimisation. Here we used it to optimise the composition of a commonly used synthetic growth media to obtain a media capable to support growth to high cell densities. We performed two rounds of starting point exploration and optimisation in the direction of steepest ascent. Interestingly, the optimisation mainly increased the supplemented aa and vitamin addition, while increasing the amount of glucose and nitrogen to a lesser level. The method maximises a target value. It is therefore crucial, that this value encompasses all desired properties. In our case, we realised that optimisation solely for cell density leads to slow growth and large cells. We therefore optimised for a combination of growth rate and cell density, while using an increase in cell size as an exclusion criteria. The method is therefore well suited for any process where the goal can be well quantified and written as a target value.

Importantly, cells grown as a small batch in HCD medium showed an increased secretion of amylase. The increase is more than the tenfold increase expected if it is purely based on cell numbers. This is probably due to a more equal growth of every member of the population as well as an increase of protein production per time as the cells grow faster in HCD than in SD. We therefore successfully miniaturized a previously hard to scale down assay. As the different HCD media are based on the common ingredients media found in every yeast lab, we expect it to be of instantaneous use when large cell numbers are required, but cultivation is only feasible in small vessels.

5 Material and Methods

5.1 Yeast strains

Table 3: Yeast Strains

Strain	**Genotype**	**Reference**
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	[4]
FY4	MATa	[28]
RM11	MATa/α leu2Δ0 ura3-Δ0 HO::kanMX	[6]
W303-1B	MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15	Euroscarf
CEN.PK	MATa/α ura3-52 trp1-289 leu2-3_112 his3Δ1 MAL2-8C SUC2	[25]
FRY745	BY4741 URA3::P$_{TEF2}$ (-1-402)-CitrineA206K-Cyc1-Term	[16]
FRY746	BY4741 URA3::P$_{ADH1}$ (-1-1500)-CitrineA206K-Cyc1-Term	[16]
FRY748	BY4741 URA3::P$_{ACT1}$ (-1-520)-CitrineA206K-Cyc1-Term	[16]
FRY757	BY4741 URA3::P$_{TDH3}$ (-1-680)-CitrineA206K-Cyc1-Term	[16]

5.2 Standard media used for comparison

Standard yeast media (YPD and SD) compositions were used [22].

5.3 Stock Solutions

Table 4: HCD medium Composition

Component	**Final Concentration**
Glucose	(10x = 200 g/ℓ)
Nitrogen 1 (Monosodium Glutamate)	(50x = 50 g/ℓ)
Nitrogen 2 (aa acid supplement)	(50x = Glu = 5 g/ℓ, His = 1.75 g/ℓ, Leu = 5.5 g/ℓ, Lys = 6 g/ℓ, Met = 2 g/ℓ, Phe = 2.5 g/ℓ, Ser = 1.875 g/ℓ, Thr = 1 g/ℓ, Ura 2 g/ℓ))
Trace/Vit 1 (YNB - complex mixture)	(40x = 60g/ℓ)
Trace/Vit 2 (inositol)	(50x = 100mg/ℓ, prepare as 5000x stock solution 0.5 g/50mℓ)
Buffer Solution	(50x = 1 mol/l MES, 1 g/ℓ Ammonium sulfate, 1 g/ℓ Sodium dihydrogen phosphate adjust pH of solution to 6)
Water	

5.4 Material

All chemicals were obtained from Sigma-Aldrich (Buchs, Switzerland) unless otherwise indicated.

5.5 Growth experiments

Strains were grown overnight at 30°C in 2 mℓ of the indicated medium. The next day 10 000 or 20 000 cells were seeded in 800 $\mu\ell$ of the same medium in a 48-well FlowerPlate (without optodes) (m2p-labs, Baesweiler, Germany). The plate was covered with a gas-permeable sealing film (m2p-labs) and placed in a BioLector device (m2p labs) incubated at 30°C, with 20.95% O_2, $\geq$ 85% humidity, shaken at 1 300 rpm and biomass was measured as a function of back scatter at 600 nm every 15 minutes.

5.6 Measurement of cell size and number

100 $\mu\ell$ of cell suspension was added to 10 mℓ of PBS and cell size distribution and cell number was measure in a Z2 Coulter Counter (Beckman Coulter, Nyon, Switzerland), with a 100μm orifice. For very dense cultures cells were additionally diluted 1:10 in PBS.

5.7 Analysis of cell-cycle behaviour

Strains were grown overnight at 30°C in 2 mℓ of the indicated medium. The next day the culture was diluted 1:20 in the same medium and grown for 6-7 hours at 30°C. 600 μL of culture was serially diluted (1:2) across 8 or 12 wells of a 96-well deep well plate (Kuhner, Birsfelden, Switzerland). The plate was covered with a gas-permeable lid and placed in an ISF1-X (50 cm diameter) Kuhner shaker and incubated at 30°C shaken at 330 rpm overnight. The next day 100 $\mu\ell$ of cells were transferred to a 96-well MTP (Greiner Bio One, Kremsmünster, Austria) diluted with PBS and fluorescence of strains was analysed using a Fortessa flow analyser (Becton Dickinson, Allschwil Switzerland). For DNA staining cells were fixed by resuspending in 500 $\mu\ell$ of cold 70% ethanol and incubated 15 minutes at RT. Cells were harvested and resuspended in 500 $\mu\ell$ of 2 mg/mℓ Proteinase K in 50 mM Tris pH 7.5 and incubated for 50 minutes at 50°C. Cells were harvested and resuspended in 500 $\mu\ell$ 0.2 mg/mℓ RNaseA in 50 mM Tris pH 7.5 for 4 hours at 37°C. Cells were harvested and resuspended in 500 $\mu\ell$ FACS buffer (200 mM NaCl, 78 mM $MgCl_2$, 200 mM Tris pH 7.5) and stored at 4°C. Before analysis 100 $\mu\ell$ of cells were added to 1 mℓ of 1 μM Sytox Green (Thermo Fisher, Basel, Switzerland) in 50 mM Tris pH 7.5.

5.8 Amylase secretion assay

BY4741 harbouring pYapAmyGPD [24] expressing α-amylase were grown overnight at 30°C in 2 mℓ of the media lacking uracil. The next day the culture was diluted 1:20 in the same medium and grown for 6-7 hours at 30°C. 600 μL of

culture was serially diluted (1:2) across 12 wells of a 96-well deep well plate (Kuhner, Birsfelden, Switzerland). The plate was covered with a gas-permeable lid and placed in an ISF1-X (50 cm diameter) Kuhner shaker and incubated at 30°C shaken at 330 rpm overnight. The next day cells were pelleted for 3 min, 2 000×g in an Eppendorf 5920R centrifuge (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). 50 $\mu\ell$ of supernatant was transferred to a 96-well multi-well plate (Greiner Bio One, Kremsmünster, Austria). The amylase assay was performed using the Amylase Activity Assay Kit from Sigma (MAK009), all steps were performed according the manufacturer's instructions. OD405 was measured using a Tecan Infinite M Nano Absorbance Reader (Tecan Group Ltd., Maennedorf, Switzerland).

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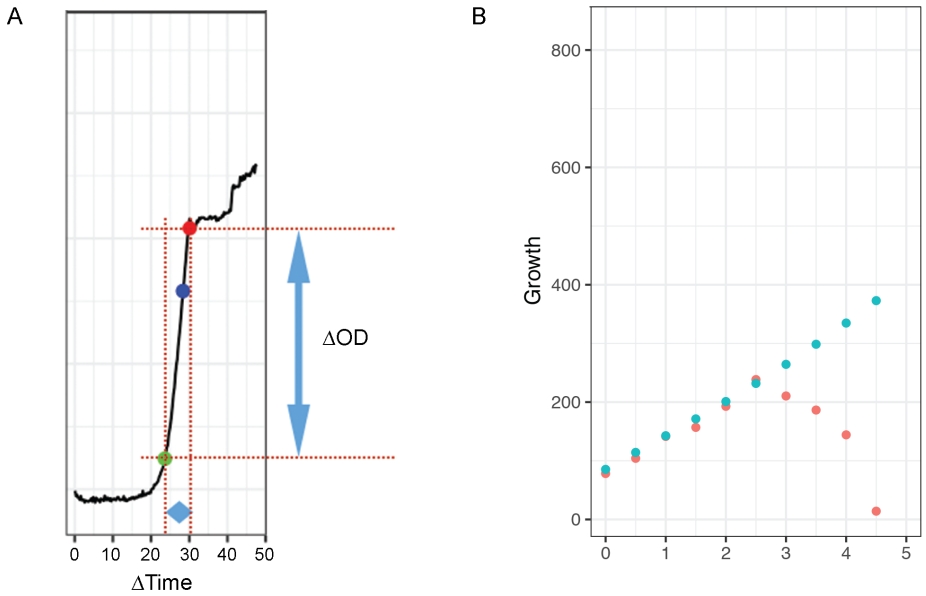

Figure 1: (A) Definition of response functions. For the first iteration, we optimised the increase ΔOD in optical density from beginning of growth (green point) to onset of the diauxic shift (red point). For the second iteration, we subtracted a multiple of the corresponding duration to account for growth speed. (B) Comparison of predicted (green) and measured (red) increase in optical density during growth along the first gradient of steepest ascent.

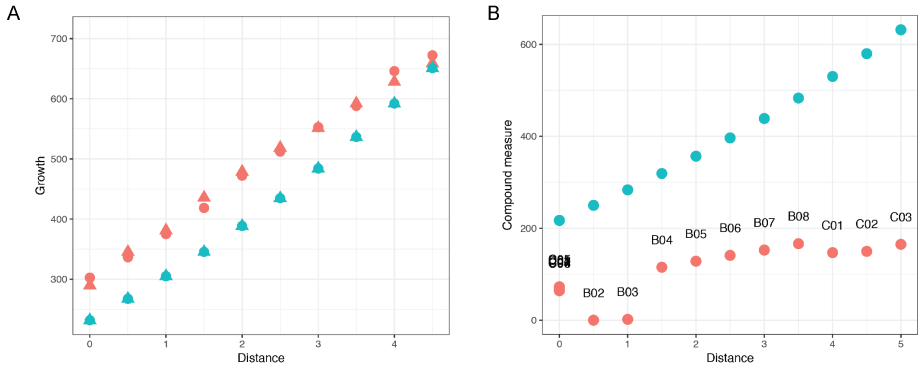

Figure 2: (A) Comparison of predicted (green) and measured (red) increase in optical density during growth along the second gradient of steepest ascent. The two point shapes correspond to two independent replicates of exploration and gradient pursuit experiments. (B) Comparison of predicted (green) and measured (red) responses of increase in optical density adjusted for duration ("compound measure") along modified gradient. Numbers denote well in the 48-well plate.

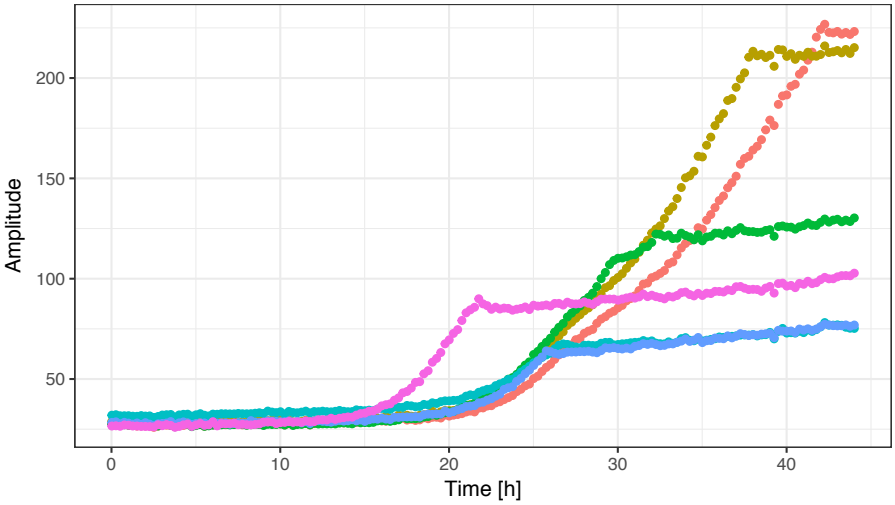

Figure 3: Comparison of growth curves for different media. Light blue and blue: original medium before any optimisation. Green: our starting medium after initial manual optimisation of components. Red: final medium after optimisation for increase in optical density. Orange: final medium after optimisation for increase in optical density and duration. Pink: YPD for comparison.

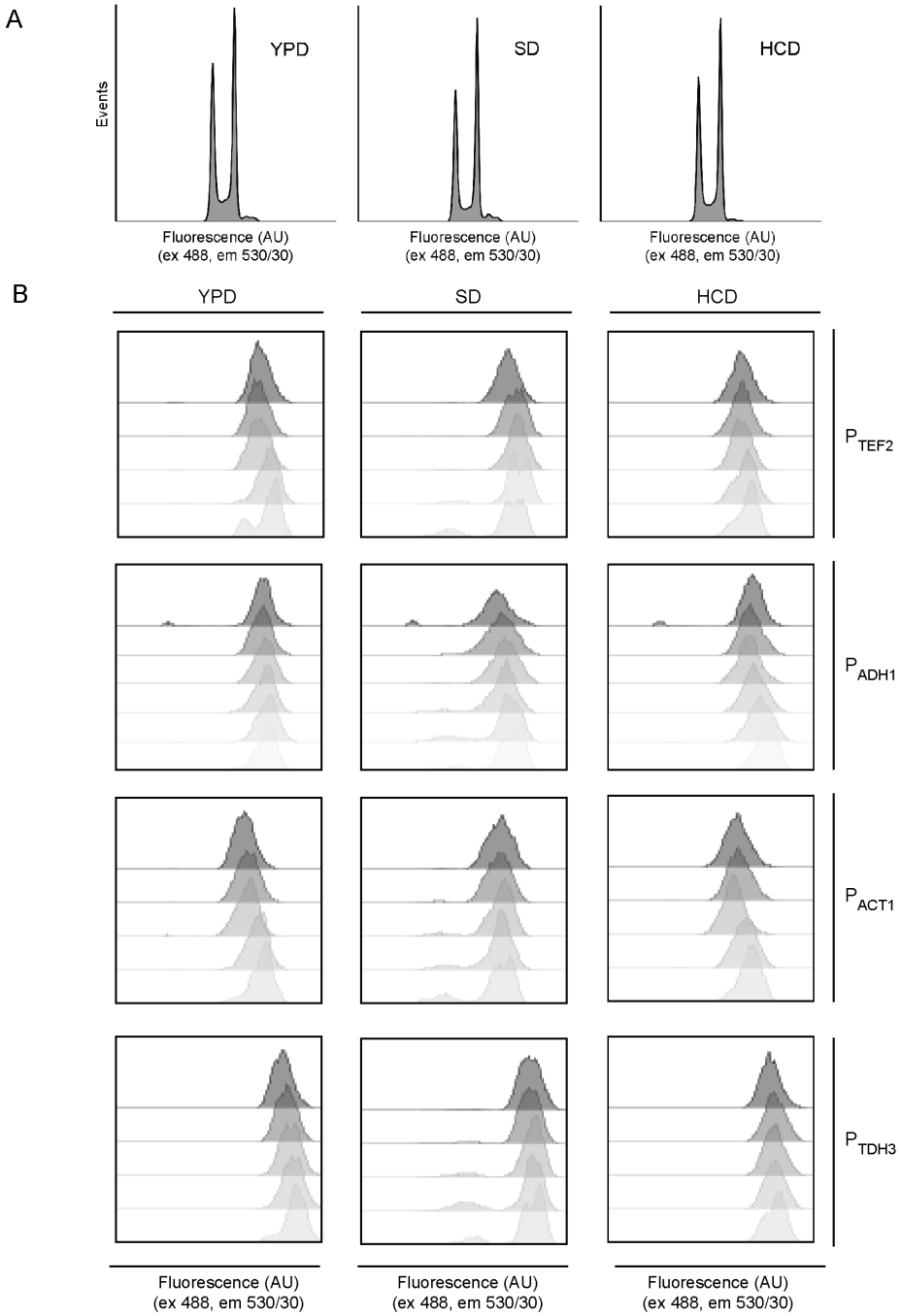

Figure 4: Cells grown in HCD medium exhibit normal cell cycle behaviour. Cells from an exponentially growing culture were serially diluted 1:2 and seeded in a deep-well plate and incubated at 30°C at 300rpm in the indicated medium. (A) Cells in mid-log phase were harvested, fixed and the DNA stained with Styox green. DNA content (which is proportional to fluorescence) is shown. (B) Cells expressing citrine from the indicated promoters were harvested from different growth phases (early log to stationary; top to bottom) and fluorescence was examined.

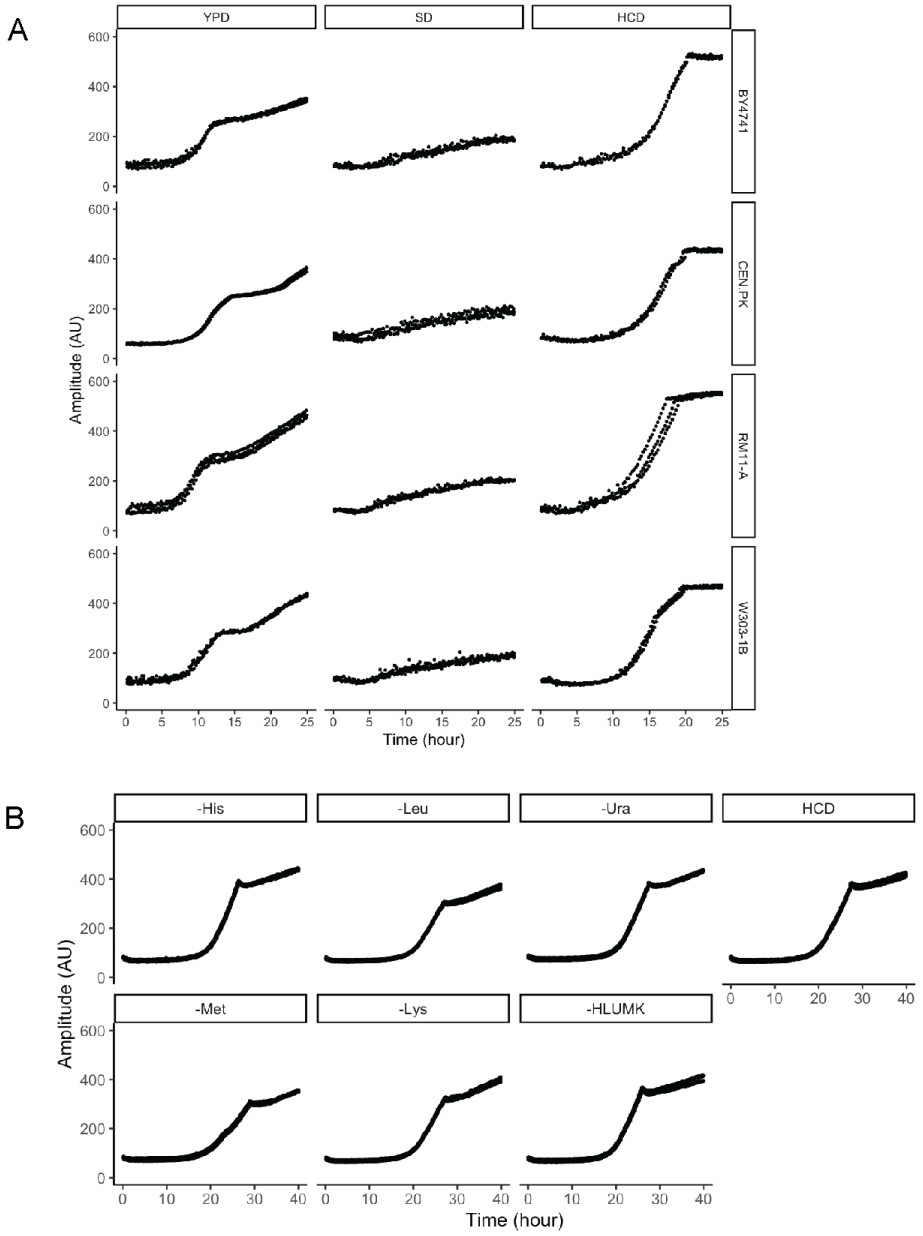

Figure 5: HCD is a versatile medium. 10 000 BY4741 cells (A) or 10 000 cells of the indicated strains (B) were seeded in a deep-well plate an incubated at 30°C at 300rpm, in the indicated medium. Individual data points are plotted from at least 4 replicates.

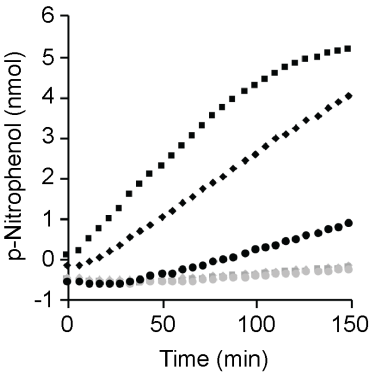

Figure 6: HCD facilitates miniaturisation of biochemical assays. Cells, expressing α-amylase from the plasmid (pYapAmyGPD), from an exponentially growing culture were serially diluted 1:2 and seeded in a deep-well plate an incubated at 30°C at 300rpm in HCD-ura (black) or SD-ura (grey) overnight. The culture supernatant was sampled from different growth phases (early log (circles), late-log (squares), or stationary (diamonds) and α-amylase secretion was examined as function of the cleavage of ethylidene-pNP-G7 to yield p-Nitrophenol (OD_{405}).