Development and optimisation of a defined high cell density yeast medium

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Abstract
Saccharomyces cerevisiae cells grown in a small volume of chemically defined media neither reach the desired cell density nor grow at a fast enough rate to scale down the volume and increase the sample number of classical biochemical assays, as the detection limit of the readout often requires a high number of cells as an input. To ameliorate this problem, we developed and optimised a new high cell density (HCD) medium for S. cerevisiae. Starting from a widely used synthetic medium composition, we systematically varied the concentrations of all components without the addition of other compounds. We used response surface methodology to develop and optimise the five components of the medium: glucose, yeast nitrogen base, amino acids, monosodium glutamate, and inositol. We monitored growth, cell number, and cell size to ensure that the optimisation was towards a greater density of cells rather than just towards an increase in biomass (i.e., larger cells). Cells grown in the final medium, HCD, exhibit growth more similar to the complex medium yeast extract peptone dextrose (YPD) than to the synthetic defined (SD) medium. Whereas the final cell density of HCD prior to the diauxic shift is increased compared with YPD and SD about threefold and tenfold, respectively. 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 they allow selection for all common yeast auxotrophic markers.

KEYWORDS
assay miniaturisation, cell-to-cell variability, growth medium, optimisation, protein production, protein secretion, response surface methodology, Saccharomyces cerevisiae

1 INTRODUCTION

The yeast Saccharomyces cerevisiae is a broadly used eukaryotic model organism in basic research as well as an important tool in biotechnology (Botstein & Fink, 2011). In contrast to many other model organisms, yeasts offer comparatively simple genetic manipulation using either plasmid-based expression or genome modifications (Gietz & Woods, 2006). 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 medium for auxotrophic selection (Gnügge & Rudolf, 2017). Most commonly, a defined growth medium uses glucose as its only carbon source with concentrations varying between 10 and 60 g/L. Additionally, a nitrogen source—in most cases ammonium sulphate—phosphate 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 efficient growth of certain lab strains (Hanscho et al., 2012). Lastly, the most common defined media have a set of amino acids added, whose composition and concentrations vary among recipes and laboratories (Hanscho et al., 2012). Although these media have
A crucial step towards miniaturisation of all biochemical assays is the increase in the amount of starting material by increasing the cell density at the diauxic shift, without dramatic changes to the cell physiology. Preferably, this should be achieved by optimisation of the concentrations of the ingredients of commonly used defined growth medium and keeping the growth rate, cell size, and cell-cycle timing similar to the established standard conditions to not dramatically alter the cellular physiology. Such a medium would aid to lift the readout above the detection limit and thereby allow more labs to perform biochemical assays using standard Society for Biomolecular Screening plates.

Optimising growth conditions is a standard procedure to make biotechnological production processes economically viable, mainly as more biomass per volume requires less production capacity, generates less waste, and gives more yield per volume (Westman & Franzén, 2015). However, most of these optimisations start from a complex medium and then add buffer substances, carbon sources, and vitamins to increase biomass (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2013; Wang, Zhang, & Tan, 2010; Mendes-Ferreira et al., 2010). The resulting media change the cellular physiology and do not allow for the use of auxotrophic markers and are therefore ill-suited for basic research. Only few media were optimised for use with auxotrophic markers and allow balanced growth during the exponential phase. An interesting example of this type increases secretion of proteins but does not increase the cell density by adding additional amino acids to the classical defined synthetic medium while keeping the rest of the ingredients constant (Wittrup & Benig, 1994; Görgens, Van Zyl, Knoetze, & Hahn-Hägerdal, 2005).

Response surface methodology (RSM) is a statistical technique for iterative improvement of a process by sequential experimentation and is a common technique for optimising (bio-)chemical and industrial processes (Box & Hunter, 1957; Box, Hunter, & Hunter, 2005, Supplementary Text for detailed explanation). The response surface describes the expected response of the process (such as the cell density) for any specific values of a set of explanatory variables (such as the concentration of components of a medium). The goal of RSM is to find a (local) optimum, that is, a combination of concentrations of components that maximise the response. In general, the response surface is unknown, and RSM iterates between exploration around a defined point to locally approximate the surface and measure the response along the predicted path of steepest ascent. In a first set of experiments, the response surface is explored in the vicinity of an initial medium composition (Figure 1a). For this, a set of new compositions is designed by systematically and simultaneously altering the amounts of all components, and the resulting responses are measured. A regression model is then fitted to the measurements to locally approximate the response surface and to calculate the direction that yields the steepest ascent on this surface (the canonical path). In a subsequent set of experiments, the responses are measured along this path at different distances from the initial medium (Figure 1b). Typically, the condition with highest response is then taken as the new intermediate medium composition, and the two steps are repeated (Figure 1b). This procedure is continued until the resulting response is deemed optimal, for example, no increase in the response is gained, the resulting response is deemed sufficiently high, or the experimental resources are exhausted.

Here we present an optimised synthetic well-defined medium allowing for growth of S. cerevisiae lab strains to high cell densities (HCDs). Only the composition of the commonly used synthetic defined (SD) medium is changed, and it is therefore usable with common auxotrophic or drug resistance markers. We term it HCD medium. It yields tenfold more cells at the diauxic shift compared with SD medium and threefold compared with YPD. Importantly, it shows balanced growth during 12 hr of cultivation as scored by growth rate and the cell-to-cell variability of growth rate sensitive reporter genes. The medium was obtained by using two iterations of RSM-based designs. We only optimise the concentrations of the ingredients of the commonly used synthetic minimal media, with monosodium glutamate as a nitrogen source to allow for use of drug resistance marker genes. The RSM-based optimisation lead to nonintuitive changes to the medium composition: the medium was not only optimised for more glucose, but the amount of amino acids and YNB was also dramatically increased, whereas the nitrogen source, monosodium glutamate, was only slightly increased. We confirmed that the media are broadly applicable, using a series of auxotrophic markers as well as lab strains. HCD media can be used to amend and miniaturise biochemical assays, such that they can be performed in 96-well deep-well plates. Additionally, the media are favourable for protein production and especially protein secretion,
FIGURE 1 Overview of response surface methodology methodology. (a) Central composite design for three components. Star: centre point, circles: factorial points, and diamonds: axial points. For clarity, axial points for third component are not shown. After coordinate transformation, centre point is at origin and factorial points are \((±1, ±1, ±1)\); new coordinates are shown for select points. (B) Sequential optimisation using response surface method. i: Original medium components with concentrations \(c_1\) and \(c_2\), respectively, lead to response surface shown as thin solid lines with a maximum response denoted by a triangular point. The starting composition is \(c_∗_1\) and \(c_∗_2\), shown as a black point. ii: In the first exploration, response surface methodology shifts and scales the coordinate system to \(x_1\) and \(x_2\) (grey lines). The central composite design requires measuring the response at the indicated round points. iii: dashed lines show the approximate response surface on the basis of the locally fitted model. The central composite design and the true surface are shown in grey. Following the canonical path (solid line) yields the intermediate optimum at the star-shaped point. iv: the procedure is repeated from the new intermediate optimum. The new local approximation predicts a maximum upward and to the left of the actual optimum, but the slightly curved canonical path still approaches the actual maximum. The procedure can be repeated again if required

as shown by an increased and constant per cell expression of the test protein amylase during the whole growth phase.

2 | MATERIAL AND METHODS

2.1 | Yeast strains

Different strains were used, and Table 1 gives an overview of the source and the relevant genomic modification.

2.2 | Standard media recipes

YPD medium was composed of 30-g/L glucose, 10-g/L yeast extract (Difco), and 20-g/L bacto peptone (Difco). SD was composed of 0.17% YNB without amino acids or ammonium sulphate (BD Biosciences, Germany), 0.5% ammonium sulphate as nitrogen source, and 2% glucose. Additionally, 20-mg/L Arg, 100-mg/L Asp, 100-mg/L Glu, 20-mg/L His, 300-mg/L Ile, 60-mg/L Leu, 30-mg/L Lys, 20-mg/L Met, 50-mg/L Phe, 375-mg/L Ser, 200-mg/L Thr, 40-mg/L Trp, 30-mg/L Tyr, 150-mg/L Val, and 20-mg/L Ura were added. The yeast extract media were composed of 30g/L glucose and 5g/L yeast extract.

2.3 | Stock solutions

The individual chemicals for the medium optimisation were provided as concentrated stock solutions. The different pools of chemicals and the individual concentrations are given in Table 2. All components were filter sterilised, except for YNB, which was autoclaved. We have noticed that 60g/L of YNB is at the saturation point and that the solubility is increased at lower pH; however, we recommend using a lower concentration for preparing the YNB stock (Table 3).

2.4 | Chemicals

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

2.5 | 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 μl of the same medium in a 48-well FlowerPlate (without optodes; m2p-labs, Bäsweiler, 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 ambient O₂ concentration (measured 20.95%), ≥ 85% humidity, and shaken at 1,300 rpm, and biomass was measured as a function of back scatter at 600 nm every 15 min.

2.6 | Measurement of cell size and number

One hundred microlitres of cell suspension was added to 10 ml of phosphate buffered saline (PBS), and cell size distribution and cell number were measured in a 22 Coulter Counter (Beckman Coulter, Nyon, Switzerland), with a 100-μm orifice. Very dense cultures cells were additionally diluted 1:10 in PBS.

2.7 | 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
TABLE 1 Yeast strains

| Strain         | Genotype              | Reference                        |
|---------------|-----------------------|----------------------------------|
| Sc BY4741     | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Brachmann et al. (1998)          |
| Sc FY4        | MATa                  | Winston, Dollard, and Ricupero-Hovasse (1995) |
| Sc RM11       | MATα leu2Δ0 ura3Δ0 HO::kanMX | Brem, Yvert, Clinton, and Kruglyak (2002) |
| Sc W303-1B    | MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 | Euroscarf |
| Sc CEN.PK     | MATα ura3-52 trp1-1289 leu2-3,112 his3Δ1 MAL2-8C SUC2 | van Dijken et al. (2000) |
| Pp X-33       |                       | ThermoFisher Scientific          |
| Sp 972        | h-                    | Leupold (1950)                   |
| Sc FRY745     | BY4741 URA3::P₆₆₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋‧chezium pombe. |

| Strain         | Genotype              | Reference                        |
|---------------|-----------------------|----------------------------------|
| Sc FRY746     | BY4741 URA3::P₆₆₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋˓<content>grown for 6–7 hr at 30 °C. Six hundred microlitres 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 setting) Kuhner shaker and incubated at 30 °C. Six hundred microlitres 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 xg in an Eppendorf 5020R centrifuge (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). Fifty microlitres of supernatant was transferred to a 96-well multiwell 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. OD at 405 nm was measured using a Tecan Infinite M Nano Absorbance Reader (Tecan Group Ltd., Männedorf, Switzerland).

2.8 Amylase secretion assay
BY4741 cells harbouring pYapAmyGPD [Tyó, Liu, Petrano, & Nielsen, 2012] expressing α-amyrase were grown overnight at 30 °C in 2 mL of media lacking uracil. The next day, the culture was diluted 1:20 in the same medium and grown for 6–7 hr at 30 °C. Six hundred microlitres 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 xg in an Eppendorf 5020R centrifuge (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). Fifty microlitres of supernatant was transferred to a 96-well multiwell 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. OD at 405 nm was measured using a Tecan Infinite M Nano Absorbance Reader (Tecan Group Ltd., Männedorf, Switzerland).

2.9 Plasmid rescue and transformation
Overnight cultures of BY4741 harbouring FRP2061 (a 13-kb plasmid with a dual promoter Kanamycin cassette for resistance in both Escherichia coli [kanamycin] and S. cerevisiae [G418]) were diluted 1:25 in 1 mL in deep-well plates (Kuhner, Birsfelden, Switzerland). The plate was covered with a gas-permeable lid and placed in an ISF1-X (50-mm diameter setting) Kuhner shaker and incubated at 30 °C shaken at 330 rpm for 8 hr in the indicated media with 340-μg/ml G418. Samples were harvested and stored at 4 °C in 50-μl spheroplasting solution (per 1-ml 1-M sorbitol, 6-μl 1-M DTT, and 15-μl 10-mg/ml 100T zymolyase) overnight. The next day, the samples were incubated 2 hr at 37 °C, and spheroplasting was verified microscopically. Fifty microlitres Qiagen P1 buffer was added, followed by 50-μl Qiagen P2 buffer, and 70-μl Qiagen N3 buffer (Qiagen, Hilden, Germany). The sample was transferred to 96-well polyvinylidene fluoride plate, stacked on top of a 96-well glass filter plate, on top of 96-well collection plate, and spun 3,000 xg, 10 min in an Eppendorf 5020R centrifuge (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The

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**Table 2**: Stock solution composition used throughout the optimisation

| Component | Concentration (g/L) |
|-----------|---------------------|
| Glucose   | 200                 |
| Nitrogen  |                     |
| Monosodium glutamate | 50          |
| Amino acid mix |                  |
| His       | 1.75                |
| Leu       | 5.5                 |
| Lys       | 6                   |
| Met       | 2                   |
| Phe       | 2.5                 |
| Ser       | 1.875               |
| Thr       | 1                   |
| Ura       | 2                   |
| Trace/Vit 1 | YNB without aa or (NH₄)₂SO₄ | 60 |
| Trace/Vit 2 | Inositol             | 0.1 |
| 50X Buffer | pH = 6               | 195.2 |
| 1-M MES   | 195.2               |
| Ammonium sulphate | 1            |
| Sodium dihydrogen phosphate | 1        |
| Water     |                     |
Optimisation using experimental design

To explore the response surface around our starting medium, we measured cell growth for each of the 32 design points using a BioLector growth reader (Supplementary Figure S1). We used the increase in amplitude (or optical density [OD]) $\Delta$OD (Figure 2b) from the start of growth to the beginning of the diauxic shift as the response that we aimed to maximise. This gives a crude but sufficient approximation of the increase in cell number. Six out of twenty-six conditions indeed showed an increase in biomass, and seven showed a discernible decrease. The remaining 13 showed similar growth as the starting medium.

After performing the exploration (Supplementary Figure S1), we calculated the path of steepest ascent and measured the response along it (Supplementary Table 1). We found that the predicted growth versus the measured growth matched well for the first six points along the canonical path (Figure 2c), after which the approximation of the response surface breaks down and the measured growth starts to decrease. Therefore, we took the sixth point as our intermediate optimum; it corresponds to the composition shown in row “Intermediate” of Table 4. A comparison of the growth curves between the starting medium and this optimum of this first round of RSM is shown in (Figure 2d). Interestingly, “Intermediate” showed a threefold increase in the amino acid pool, a slight increase in MSG, and a decrease in glucose and YNB compared with our starting condition. Six out of twenty-six conditions indeed showed an increase in biomass, and seven showed a discernible decrease. The remaining 13 showed similar growth as the starting medium.

In order to ensure that the direction of the optimisation was indeed towards more cells and not simply towards more biomass, we checked if the cells behaved normally with regard to cell size and measured cell size at the end of growth in the BioLector using a Coulter counter. We found no indication of abnormal cell sizes up to the “Intermediate” condition and a slight increase thereafter. We

### TABLE 3

| Component | Concentration (g/L) | Millilitre added per litre of HCD |
|-----------|---------------------|----------------------------------|
| 5x glucose (30%) | 300               | 200                              |
| 10x MSG     | 45                 | 100                              |
| 5x amino acid mix |                | 200                              |
| His        | 1.05               |                                  |
| Leu        | 3.3                |                                  |
| Lys        | 3.6                |                                  |
| Met        | 1.2                |                                  |
| Phe        | 1.5                |                                  |
| Ser        | 1.13               |                                  |
| Thr        | 0.6                |                                  |
| Ura        | 1.2                |                                  |
| 4x YNB*    | 48                 | 250                              |
| 100x Inositol | 0.180           | 10                               |
| 50x Buffer pH = 6 | 1.200           |                                  |
| MES        | 195.2              |                                  |
| (NH4)2SO4  | 1                  |                                  |
| NaH2PO4    | 1                  |                                  |
| Water      | 220                |                                  |

Abbreviations: HCD, high cell density; YNB, yeast nitrogen base. *Without aa or (NH4)2SO4.
FIGURE 2  Utilisation of response surface methodology to optimise media for increase in optical density (OD). (a) A defined yeast minimal medium (Table 4 “Starting”) was used as the starting condition and centre point for response surface methodology; six replicates are shown. (b) For the first iteration, the increase (ΔOD) in OD (amplitude) from the beginning of growth (triangle) to onset of the diauxic shift (diamond) was optimised. (c) Comparison of predicted (grey) and measured (black) increase in OD (amplitude) during growth along the first gradient of steepest ascent. (d) Comparison of growth of BY4741 cells in the starting conditions (grey) and the optimal conditions (black) after the first round of optimisation.

TABLE 4  Media composition throughout the optimisation. First three rows: initial medium composition and the low and high points for the response surface method. Next row: intermediate optimum after first iteration. Last row: final optimum.

| Medium     | Glucose (%) | MSG (mg/ml) | AA pool | YNB (mg/ml) | Inositol (μg/ml) |
|------------|-------------|-------------|---------|-------------|------------------|
| Starting   | 4           | 2           | 1x      | 12          | 1                |
| Low        | 2           | 1           | 0.5x    | 8           | 0                |
| High       | 6           | 3           | 2x      | 16          | 2                |
| Intermediate| 3           | 2.7         | 3.3x    | 10.8        | 1.4              |
| Final      | 6           | 4.5         | 6.0x    | 12          | 1.8              |

Abbreviations: YNB, yeast nitrogen base.

...also did not find any obvious correlation between cell size profile and a specific medium component, as was reported for inositol (Hanscho et al., 2012), suggesting that it is a combination of several components that affect cell size distribution.

Using the “Intermediate” composition as our new starting point, we iterated the two steps of response surface exploration and followed the canonical path again and obtained a second, increased response shown in supplementary Table 2. To ensure reproducibility of our results, we additionally performed two independent replicates of the exploration and subsequent experiments to pursue the direction of steepest ascent. Both replicates are in excellent agreement, as are the predicted and experimental responses (Figure 3a). The vertical shift between predictions and measurements is likely due to 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 associated with slower growth and increased cell size (Figure 3b,c), suggesting that this second iteration of the optimisation was focused towards increasing total biomass rather than total cell number. In order to see if we could optimise for higher cell numbers, we repeated the calculation of the canonical path from the data of the second exploration. We used an adapted response function $y = \Delta OD - 2 \cdot \Delta t$ to include the time $\Delta t$ elapsed between initiation of growth and reaching the diauxic shift as a crude measure for growth rate (Figure 2b). This new response function favours large increases in OD but additionally penalises long growth intervals. We verified that this steepest ascent prediction indeed follows a desired path by checking that no conditions in the vicinity of the path showed a decreased maximum growth, growth rate, or increased cell size.

We then measured the response of the 10 predictions shown in supplementary Table 3 using the BioLector and found a clear increase in growth (Figure 3d). Up until a distance of 3.0 from the centre point, maximal biomass increased and cell size and growth rate stayed the same (Figure 3e,f). From distance 3.5 on, the compound...
FIGURE 3  Comparison of gradient pursuit for optical density (OD) only or OD and time. (a) Comparison of predicted (grey) and measured (black) increase in OD (amplitude) during growth along the second gradient of steepest ascent. The two point shapes correspond to two independent replicates of exploration and gradient pursuit experiments. (d) Comparison of predicted (grey) and measured (black) responses of increase in optical density adjusted for duration ("compound measure") along modified gradient. The corresponding growth curves of BY4741 cells are shown in Panels b and e (ranging from grey to dark blue along the gradient of steepest ascent). (c, f) The cell size of BY4741 cells from the corresponding logarithmically growing cultures was measured using a Coulter counter. The empirical cumulative distribution function of the measured cell size of the optimisation on the basis of OD (c) or OD and time (f).

measure, the cell size or both were less favourable than at distance 3.0. Similar to the initial second path, we observed that the predicted responses along the new path were overly optimistic. Nevertheless, the measured responses followed a clear upward trend, indicating that the corresponding medium composition leads to higher cell numbers. The medium at distance 3.0 approximately doubled the amount of glucose, MSG, amino acid pool, and inositol and only slightly increasing YNB compared with the starting point “Intermediate.”

We stopped the optimisation after this second iteration with modified response and slightly modified the composition towards distance 2.5 to make it easier to pipette at the bench. We named the new medium HCD for high cell density medium. Its composition is shown in Table 5, and the stock solutions used in the optimisation are given in Table 2.

3.2  Cells grown in HCD medium exhibit normal physiology

HCD medium achieves cell numbers of $1.7 \times 10^8$ cells/ml at the end of the exponential phase of growth, a tenfold increase compared with the $1.6 \times 10^7$ cells/ml for SD and a threefold increase compared with the $6.0 \times 10^7$ cells/ml for YPD. A direct comparison of typical growth curves and the cell size distribution for the three media is shown in Figures 4A,B and supplementary Figure S2. It shows similar growth rates of HCD and YPD, while extending the balanced growth phase from 5 to 12 hr and that the cumulative cell size distribution of strains grown in three different media is almost identical.

To confirm that cells grown in HCD medium exhibit normal cell-cycle

| Component | Final concentration |
|-----------|---------------------|
| Glucose   | 6%                  |
| MSG       | 4.5 mg/ml           |
| YNB       | 12 mg/ml            |
| Inositol  | 1.8 μg/ml           |
| AA pool   |                     |
| 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           |
FIGURE 4  Characterisation of high cell density (HCD) media. (a) Comparison of the final optimised media (HCD; blue) with the initial media (black) and yeast extract peptone dextrose (YPD; grey). (b) The cell size of BY4741 cells from logarithmically growing cultures was measured using a Coulter counter. The empirical cumulative distribution function of the measured cell size of synthetic defined (SD; black), YPD (grey), and HCD (blue) is shown. (c, d) Cells from an exponentially growing culture were serially diluted 1:2 and seeded in a deep-well plate and incubated at 30°C at 300 rpm in the indicated medium. (c) Cells in midlog phase were harvested and fixed and the DNA stained with Sytox green. DNA content (which is proportional to fluorescence) is shown. (d) Cells expressing citrine from the indicated promoters were harvested from different growth phases (early log to stationary; top to bottom), and fluorescence was examined.

behaviour, we first looked at DNA content in logarithmically growing cultures by staining the DNA with Sytox Green (Figure 4c). We found that cultures grown in HCD medium exhibit similar ratios of cells in G1 and G2/M as SD and YPD, with a similar number of cells in S phase as
FIGURE 5  High cell density is a versatile medium. Ten thousand cells of the indicated strains (a) or 10'000 BY4741 cells (b) were seeded in a deep-well plate and incubated at 30° C at 300 rpm in the indicated medium; growth in high cell density media is shown in grey for comparison. Individual data points are plotted from at least three replicates.

YPD. This indicates that cells grown in HCD behave similarly to cells grown in YPD.

We further evaluated the cellular physiology by examining the activity of metabolic or growth rate regulated promoters during different phases of growth. We used a set of well-characterised, constitutive promoters: $P_{TEF2}$, $P_{ADH1}$, $P_{ACT1}$, or $P_{TDH3}$, driving the expression of the fluorescent reporter, citrine (Ottoz et al., 2014; Figure 4d). We scored the respective expression levels using flow cytometry and interpret increases in cell-to-cell variation as a measure for differences in cellular physiology. We found that for all four promoters, expression at different cell densities in HCD and YPD media leads to a unimodal distribution, except for $P_{TEF2}$ that shows a remarkable bimodal expression peak at the diauxic shift in YPD and to a lesser extent in HCD.

In contrast, all promoters show the tendency of bimodal expression in SD medium.

It is important that a medium is not only specific for a single strain background, for example, the background in which the optimisation was performed. Therefore, we repeated the growth curve analysis for a set of common lab strain backgrounds and assessed their behaviour in HCD in comparison with YPD and SD. We tested CEN.PK, W303-1B, and the wild isolate RM11 (Brem, Yvert, Clinton, & Kruglyak, 2002). For W303-1B and CEN.PK it was necessary to supplement HCD with 400-μg/ml adenine and/or 400-μg/ml tryptophan. Akin to the S288C derivative BY4741, all strains exhibited similar growth to YPD, with extended growth phase and higher ODs prediauxic shift (Figure 5a). W303-1B shows a premature kink in the growth rate, which most likely could be alleviated by optimising the Ade or Trp dosage.
FIGURE 6  High cell density facilitates miniaturisation of assays. (a) BY4741 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 and incubated at 30 °C at 300 rpm 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). (b) BY4741 cells containing a shuttle vector harbouring kanMX were grown in the indicated media with 200-μg/mL G418, and plasmid was isolated and transformed into Escherichia coli. The number of colonies obtained from eight independent plasmid isolations and subsequent transformations was determined (black points), and the mean (red points) and standard deviation (red lines) are indicated.

Additionally, we tested whether the commonly used Schizosaccharomyces pombe and Pichia pastoris strains h972 and X-33 can grow in HCD. To this end, we compared the cell number at the end of the growth phase in HCD and the respective complex standard medium. S. pombe grew to more than twice the cell density compared with YES (2.4×10⁸ vs. 8.5×10⁷), whereas P. pastoris grew to a lower density in HCD compared with YPD (8.8×10⁹ vs. 1.2×10⁹). In both cases, the reported cell density for a synthetic defined media is tenfold lower than the complex media we used.

We next wanted to confirm that HCD medium can be used with auxotrophic markers, which are essential tools in yeast research. They are used to maintain plasmids and to select for genomic integrations and can vary among strain backgrounds. As such, we examined modified HCD media lacking specific amino acids and grew the prototrophic strain FY4 in HCD lacking one amino acid supplement at a time (denoted HCD-aa) or lacking amino acids corresponding to several common auxotrophic markers (denoted HCD-HLUMK) in the BioLector (Figure 5b). In general, the HCD dropout media showed only minimal deviation from the fully supplemented growth with the exception of medium lacking methionine (HCD-met), which grows slower, but still reaches an HCD. HCD-Leu and HCD-Lys showed a premature saturation but identical growth rates throughout the growth curve.

3.3 HCD medium allows for assay miniaturisation

One of the motivations for designing a defined HCD medium was to facilitate the miniaturisation of assays such that assays classically performed in flasks could be performed in a more efficient manner using multiwell plates. As a proof of concept, we measured the secretion of α-amylase, a hard to secrete protein requiring the optimised media for detectable secretion (Wittrup & Benig, 1994; Tyo et al., 2012). α-Amylase is a relatively large protein, has an odd number of cysteines, and is glycosylated, all three characteristics disfavouring an efficient secretion. Expression of this protein therefore leads to a considerable amount of oxidative stress (Tyo et al., 2012).

We grew BY4741 cells harbouring a plasmid expressing α-amylase with a synthetic secretion signal (Tyo et al., 2012) in multiwell deep-well plates in 600 μl 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 caused by amylase cleavage of ethylidene-p-nitrophenol-α-D-maltoheptaosid (Figure 6a). 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.

We next compared the amount of plasmid that can be obtained from a yeast culture grown in the starting media and in HCD. To this end, we grew 1 ml of a 1:25 fold diluted culture for 8 hr in a 96-well deep-well plate in the inital starting media (i.e., already optimised compared with SD) and HCD. We isolated the plasmid as described in Section 2 and indirectly measured the amount of recovered plasmid by counting the colonies of E. coli cells transformed with the plasmid (Figure 6b). We found that the number of transformants correlated well with the optical densities of the input and took this as an indication that the optimised media was behaving as expected.

4 DISCUSSION

Miniaturising biochemical assays in S. cerevisiae is difficult as the commonly used strains, plasmids, and auxotrophic markers only grow in a medium with a short exponential phase reaching small cell numbers at the diauxic shift. Using RSM, we obtained an altered version of the commonly used synthetic medium with modified composition of its
ingredients. We termed the medium HCD, for high cell density. Cultures grown in HCD medium reached at least threefold and tenfold higher density at the diauxic shift compared with YPD and SD, respectively, and the balanced growth phase lasted up to 12 hr compared with 3 to 5 hr for the two standard media. HCD medium 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). Additionally, the media are suitable for other yeasts as both S. pombe and P. pastoris showed similar stationary phase cell numbers to the respective complex media. The medium also supports positive selection as shown using the kanMX marker gene.

The physiology of the cells growing in HCD medium seems normal, as their size remains similar to cells grown in standard conditions and the cell-cycle distribution is similar to YPD. 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 medium 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.

RSM is widely used in process optimisation. Here, we used it to optimise the composition of a commonly used synthetic growth medium to obtain a medium capable of supporting growth to high cell density. We performed two rounds of exploration and optimisation in the direction of steepest ascent. The method maximises a target value whose definition should reflect 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.

Although the final medium composition is composed of "more of everything," the optimisation illustrated that the composition has to be well balanced; otherwise, unfavourable changes in cellular physiology will manifest. Compared with the standard SD, the final medium is made up of three times more glucose, but roughly ninefold and twelvefold more MSG and amino acid pool, respectively. Therefore, to keep the cellular morphology and cell-cycle distributions unchanged at higher densities, the addition of glucose needed to be overcompensated by the addition of amino acids as well as an increase of YNB and inositol. Interestingly, the first iteration of the optimisation pointed in this direction by lowering the amount of glucose and YNB while increasing the amount of amino acids and inositol provided. In the second iteration the pattern was similar but less pronounced, as we initially created an increase in biomass and cell size as well as a slow down of growth by increasing everything. Lowering the rate of glucose increase while increasing the amount of YNB, MSG, and inositol corrected the optimisation to a higher cell density without increasing the cell size or distorting cellular physiology. HCD medium thus represents a finely tuned medium for balanced cell growth to high densities.

Importantly, cells grown as a small batch in HCD medium showed an increased secretion of amylase. The increase is larger than expected based on the increased cell density likely due to the more uniform 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 miniaturised α-amylase secretion, a difficult to produce protein and hence hard to scale down. Additionally, we showed that purification of plasmid DNA is feasible using a standard 96 glass filter well plate. By extension, we assume that HCD will allow for efficient protein assays obtained from small volumes grown in 96-well plates. As the different HCD media are based on the common ingredients found in every yeast lab, we expect them to be of instantaneous use when large cell numbers are required, but cultivation is only feasible in small vessels.

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