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Short Communication

Development of a chemically defined medium for *Yarrowia* yeasts using a strategy of biological mimicry ☆

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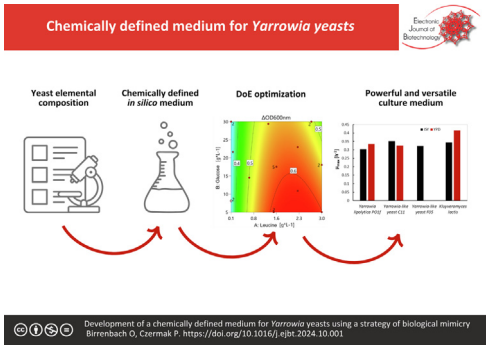
GRAPHICAL ABSTRACT



Chemically defined medium for *Yarrowia* yeasts

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ABSTRACT

Background: The efficiency of recombinant protein production in *Yarrowia lipolytica* is strongly influenced by the culture medium. Complex media have limited applications because the composition is undefined and variable, whereas chemically defined media achieve better yields and reproducibility. Defined media also increase selectivity, reduce contamination risks, and enable precise nutrient control, leading to better growth and higher productivity. We used a design of experiments approach based on the elemental composition of yeast for the arithmetic development and optimization of a defined medium for the *Y. lipolytica* auxotrophic strain PO1f.
Results: Statistically supported optimal concentrations of 10 $g*L^{-1}$ glucose and 2.29 $g*L^{-1}$ leucine enabled superior growth in the new *in silico* yeast (ISY) medium. Specific growth rates of 0.305 h^{-1} were achieved for the auxotrophic *Yarrowia* strain. Thiamine hydrochloride was a growth-limiting component, and higher concentrations increased the cell density of *Y. lipolytica* PO1f cultures by a factor of 30.

☆ Audio abstract available in Supplementary material.

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Leucine
Media composition
Thiamine
Yarrowia spp.
Yeasts

Conclusions: ISY medium was suitable not only for the cultivation of *Y. lipolytica* but also universally applicable for *Yarrowia*-like and other yeasts, achieving better growth rates and yields compared to existing chemically defined media.
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1. Introduction

The production of recombinant proteins is strongly affected by the properties of the host cell, the characteristics of the recombinant protein itself, the promoter used to drive gene expression and, above all, the composition of the culture medium. This complex interaction between diverse factors can limit product formation at several levels.

Although extensive data on the cultivation of *Yarrowia lipolytica* have already been published, the reports focus mainly on the production of native substances such as citric acid, fatty acids, single-cell proteins, proteases or related proteins such as lipases, and may not always be fully transferable to the production of recombinant proteins [1,2,3,4,5,6]. In addition, studies on the expression of recombinant proteins in this yeast species are mostly based on the use of complex media in shake-flask cultures. Most publications concerning the production of recombinant proteins in *Y. lipolytica* also report the use of the constitutive *hp4d* promoter, or the *XPR2* promoter, which requires high peptone concentrations in the culture medium for complete induction [7,8]. Complex media generally enable higher growth rates and recombinant protein yields in upstream processing [9]. However, the use of complex media containing peptones and yeast extract is suboptimal for industrial processes because the undefined composition results in pronounced batch-dependent variability and high costs [10,11]. In addition, the recombinant protein must be separated from serum and extract components during the downstream processing steps. For these reasons, the development of chemically defined media not only increases recombinant protein yields but also their consistency enabling a tightly controlled production. For example, a 400-fold increase in the yield of human interferon α2b was achieved in *Y. lipolytica* by selecting a suitable chemically defined base medium and optimizing the supplements using statistical experimental designs [10]. This increase reflects the comparison of a batch process in a stirred-tank reactor with optimized shake-flask cultures in complex medium, showing that media adaptation in combination with improved process control has a beneficial effect on process efficiency.

Another major advantage of chemically defined media is their selectivity. Due to the defined composition of the media, they can be designed to support the growth of specific organisms while inhibiting others [12]. This reduces the risk of contamination, which means that antibiotics can be avoided without jeopardizing sterility, thus saving costs and improving product quality during large-scale production [13].

Culture media are designed to reflect the elemental composition and biosynthetic capacity of particular cells [9]. Although the elemental composition of all microbial cells is similar, their biosynthetic capacity varies greatly. The composition of an efficient medium must therefore be adapted for each host organism. In addition to regulatory and process-related advantages (such as fewer requirements for raw material approval, simplified product purification, and less influence on measurements), chemically defined media allow the precise control and adjustment of individual nutrients [14,15]. Accordingly, consumption rates and the concentration-dependent influence of individual media components can be predicted [16]. Process engineering by adapting the nutrient supply then enable stronger and, above all, controllable cell growth and lead to higher cell productivity [15,16].

Here, we describe the development of a selective, chemically defined medium for *Yarrowia* yeasts, which enables the investigation of new isolates such as the recently discovered *Yarrowia*-like yeasts (Y_LYs) and standard research strains such as *Y. lipolytica* PO1f [17,18]. Using biological mimicry, a strategy that optimizes cell growth by ensuring the medium contains all components at the concentrations required for biomass formation, a basic formulation was defined and adapted using a combined design of experiments (DoE) and clustered supplement approach [19].

2. Experimental

All media development experiments were carried out using *Y. lipolytica* PO1f CLIB 724, a double auxotroph derived from the wild-type strain W29 that uses leucine and uracil as selection markers. The suitability of the chemically defined *in silico* yeast (ISY) medium for other yeast strains was verified using Y_LYs C11 and F05 (Fraunhofer IME, Giessen, Germany) as well as *Kluyveromyces lactis*.

The FM22 trace element and vitamin solution were prepared as previously reported [20]. The stock solution of growth factors

Table 1
Composition of the *in silico* yeast (ISY) medium and trace element solution.

ISY medium	
Components	**Concentration [g*L^{-1}]**
Glucose	10.00
K_2HPO_4	0.87
KH_2PO_4	12.93
Sodium citrate·$2H_2O$	1.00
$CaSO_4·2H_2O$	0.52
$MgSO_4·7H_2O$	2.05
$FeSO_4·7H_2O$	0.20
$(NH_4)_2SO_4$	3.77
Uracil	0.46
Leucine	2.29
Thiamine hydrochloride	0.0002
Trace element solution	10 mL*L^{-1}
pH, adjust with 10% (v/v) KOH	5.6

Trace element solution	
Ingredients	**Concentration [g*L^{-1}]**
$MnSO_4·H_2O$	0.086
$Na_2MoO_4·2H_2O$	0.050
$CuSO_4·5H_2O$	3.000
$CoCl_2$	0.250
$ZnCl_2$	3.000
H_2SO_4	5 mL*L^{-1}

consisted of 0.2 mg*L^{-1} *para*-aminobenzoic acid, 0.4 mg*L^{-1} thiamine hydrochloride and 2 mg*L^{-1} *myo*-inositol. The final composition of the ISY medium is shown in Table 1. After preparation, the medium was passed through a 0.2-µm filter. For yeast cultivation in stirred-tank reactors, the antifoam agent Struktol J673A was added to the medium at a ratio of 1:10,000 before filtration.

The concentration-dependent effect of leucine and glucose on cell growth was investigated using a DoE approach with a full-factorial, central composite design of 21 experiments over a design space of 0.1–3 g*L^{-1} leucine and 5–30 g*L^{-1} glucose. We analyzed growth kinetics in 96-well microtiter plates, with three adjacent wells for each condition making up experimental triplicates. Non-inoculated ISY medium was used as a negative growth control. We added 150 µL ISY medium per well, with corresponding concentrations of leucine and glucose, and inoculated the media to an optical density at 600 nm (ΔOD_{600nm}) of 0.5. To minimize evaporation and to protect against contamination, each well was covered with 50 µL mineral oil. The cells were cultivated at 28°C with constant shaking, and the OD_{600nm} was measured every 15 min for 24 h in a BioTek Synergy HTX multimode reader (Agilent Technologies, Santa Clara, CA, USA).

Unless otherwise stated, cells were cultivated in 500-mL baffled flasks with a working volume of 10% inoculated to an initial ΔOD_{600nm} = 0.1 and were incubated at 28°C shaking at 250 rpm (max displacement = 25 mm) in a Multitron orbital shaking incubator (Infors HT, Bottmingen, Switzerland).

The stirred-tank reactor experiments were carried out using a Labfors 3 system (Infors HT) equipped with baffled, 7-L glass vessels. Each vessel had a maximum working volume of 5 L and contained three six-blade Rushton stirrers, a macrosparger, a pH probe and an optical probe for dissolved oxygen (pO_2). A starting volume of 3 L was used in all experiments. The temperature, stirrer speed and gassing rate were kept constant at 28°C, 800 rpm and 4.5 L*min^{-1}, respectively. The pO_2 was kept at 40% by controlling the proportion of oxygen in the supply air. The pH was maintained at 5.6 by adding 25% ammonia when required. The bioreactor was inoculated to an initial ΔOD_{600nm} of 0.1. Batch cultivation started with 10 g*L^{-1} glucose. For fed-batch experiments, the initial glucose feed concentration was 2 g*L^{-1} and feeding was started automatically when the glucose was depleted. The feeding solution consisted of ISY medium with a glucose concentration of 100 g*L^{-1}.

A biospectrometer-kinetic-photometer (Eppendorf, Hamburg, Germany) was used to determine the ΔOD_{600nm}. Samples were diluted in 0.9% NaCl to keep the absorbance range between 0.1 and 0.8 AU. The cell dry weight (CDW) was determined using an MA100Q infrared moisture analyzer (Sartorius AG, Göttingen, Germany). For this purpose, a fixed volume of 4 mL of the culture broth was centrifuged for 3 min at 16,100 × g, resuspended in 1000 µL ultrapure water, and dried at 180°C on a glass fiber sample carrier before weighing.

3. Results and discussion

3.1. In silico design of a chemically defined medium for Yarrowia lipolytica

Molar ratios of elements in the ISY medium (Table 1) were based on the elemental composition of yeast cells [21,22] with carbon as the baseline (e.g., 1:0.034:0.059:0.024 for C:N:P:S). Suitable compositions of salts were taken from known chemically defined media for yeast, such as FM22 medium adapted for *K. lactis* [20], SM2 medium for *Candida rugosa* [23], YNB medium as a basic component of many selective media, as well as media developed for *Y. lipolytica* by Kamzolova et al. [24] and Gasmi et al. [10]. Glucose as a carbon source and ammonium sulfate as a nitrogen source were used consistently in all media. The selection of the other components was based on their beneficial properties.

We used 100 mM potassium phosphate buffer to stabilize the pH and as a source of phosphate and potassium. However, the concentrations of potassium and phosphorus required for cell growth were exceeded by factors of 2.7 and 2.9, respectively, in order to achieve sufficient buffering capacity. Sulfur was also present in excess (40 mM, 13-fold more than required), due to the provision of most of the additional salts as sulfates. The growth rate of *Candida* species remains constant or even increases with increasing sulfate concentrations up to 23.92 mM [25]. As expected, the high sulfate concentration did not have a negative effect on the cells. In addition, Y_LYs are extremely tolerant to high salt concentrations [18].

Other elemental components included sodium, magnesium, calcium, iron, zinc, cobalt, manganese and molybdenum. The positive influence of iron on the formation of *Y. lipolytica* biomass is well described for low iron concentrations. Increasing the iron concentration in the medium from 6 to 10 mg*L^{-1} increased the biomass by 47% [24]. Zinc, cobalt, molybdenum and manganese are present as trace elements at much lower concentrations in the ISY medium, but are essential for metabolism as co-factors or components of enzyme complexes [26,27,28,29].

Tri-sodium citrate was required as a chelating agent [30] because the combination of bivalent, positively charged ions such as Mg^{2+} and Ca^{2+} with potassium phosphate buffer otherwise resulted in the precipitation of sparingly soluble salt complexes. At a concentration of 1 g*L^{-1} tri-sodium citrate, all medium components dissolved as anticipated. This concentration was used because higher concentrations trigger a morphological change in the cells and the formation of hyphae. These structures of dimorphic yeast are unfavorable for homogeneous cultivation in stirred-tank reactors due to their rigid, filamentous arrangement and tendency to cross-link and form mycelia.

Extensive studies of the pH-dependent growth of *Y. lipolytica* have shown an optimum of pH 5.6 for biomass formation and the induction of filamentous growth at pH 4.5 and 7 [31]. Accordingly, we selected a pH of 5.6 for the ISY medium. The pH control in the bioreactor is designed for high-cell-density cultivation, so ammonia was used as a base and additional nitrogen source. The nitrogen input via the base was already taken into account in the design of the medium. In this way, we were able to avoid cell death triggered by high ammonium concentrations [32]. The ammonium sulfate content was calculated appropriately for 4 g*L^{-1} carbon, while all other media components were designed for 20 g*L^{-1} carbon, equivalent to 50 g*L^{-1} glucose (Table 1). Uracil and leucine were needed to compensate for the auxotrophy of the *Y. lipolytica* PO1f strain.

3.2. Verification of the suitability and universality of ISY medium

To assess the suitability of the ISY medium, we compared the growth of Y_LYs, *Y. lipolytica* PO1f and *K. lactis* in ISY and YPD media. Regressions of the exponential growth curves in shake-flask cultures showed that the maximum specific growth rate of the Y_LY strains exceeded that of PO1f in ISY medium, reaching 0.305 h^{-1} (Table 2). ISY medium therefore outperforms other chemically defined media with reported maximum specific growth rates of 0.16–0.22 h^{-1} for *Y. lipolytica* [10,33]. For the PO1f strain, the growth rate in ISY medium corresponded to 85% of the growth rate in YPD medium. In contrast, the Y_LY strain C11 grew much faster in ISY medium than in the complex medium.

Confirming the universality of the ISY medium for the cultivation of related yeasts, wild-type *K. lactis* reached a maximum growth rate of 0.343 h^{-1} (0–16 h time span) which was lower than the growth rate in complex media (e.g., 0.415 h^{-1} in YPD and

Table 2
Maximum growth rates of yeast strains in ISY and YPD media. Growth rates are calculated by regression of the ΔOD_{600nm} value during exponential cell growth.

Organism	*Yarrowia lipolytica* PO1f		*Yarrowia*-like yeast C11		*Yarrowia*-like yeast F05		*Kluyveromyces lactis*	
Media	ISY	YPD	ISY	YPD	ISY	YPD	ISY	YPD
μ_{max} [h^{-1}]	0.305	0.334	0.352	0.325	0.323	n/a	0.343	0.415

0.402 h^{-1} in a food-grade agro-industrial residue medium), but comparable to growth rates achieved in other chemically defined media, ranging from 0.33 to 0.39 h^{-1} [34,35,36].

The highest ΔOD_{600nm} of strain PO1f in ISY medium was 21.3, about half that achieved in YPD medium. Glucose consumption by strain PO1f in ISY medium decreased after 19 h even though sufficient glucose remained available. Having confirmed adequate growth rates, we next attempted to adapt the culture medium for the PO1f strain and to find the components responsible for growth limitation.

3.3. Optimization of the medium for Yarrowia lipolytica PO1f

For recombinant protein production, the native *URA3* gene is often integrated as a selection marker in the double auxotrophic PO1f strain, but leucine auxotrophy still persists. To provide optimal growth conditions and ensure that auxotrophy does not limit growth, we determined the optimum leucine and glucose concentrations using statistical experimental designs.

Significant models for the prediction of ΔOD_{600nm} and growth rate were generated in the concentration ranges 0.1–3 $g*L^{-1}$ leucine and 5–30 $g*L^{-1}$ glucose. Given the limited oxygen supply in the microtiter wells covered with mineral oil, growth was naturally limited in comparison to shaking-flask cultures. However, all cultivation methods resulted in exponential growth of the strictly aerobic yeast during the period 7–14 h. In experiments with leucine concentrations of 0.1 $g*L^{-1}$, we observed a drastic reduction in the growth rate from 14 h onwards. This indicates nutrient limitation, which was not observed at leucine concentrations >0.1 $g*L^{-1}$. Leucine therefore appears to be a limiting substance in our experiments, which can also be seen in the contour diagram of the ΔOD_{600nm} as a function of leucine and glucose (Fig. 1A). The slowest cell growth is predicted for the area with the lowest leucine concentration. In *Escherichia coli* HB101[pGEc47], the biomass yield coefficient for L–leucine depends on the glucose content of the chemically defined medium [37]. Our quadratic model of cell growth (Fig. 1A) also confirms this behavior for *Y. lipolytica*, reaching higher ΔOD_{600nm} values at lower glucose concentrations.

The model for the maximum specific growth rate predicts maximum growth at a low leucine concentration paired with a low glucose concentration (Fig. 1B). This relationship was also reported for the leucine auxotrophic strain *E. coli* K12 ER2507 in order to establish optimal growth conditions for a high-cell-density fed-batch process [38]. Furthermore, leucine and glucose reduced the specific growth rate. However, leucine tolerance is higher in *Y. lipolytica* than in *E. coli*, whose growth rate was reduced by more than 50% following an increase from 0.1 to 3 $g*L^{-1}$ leucine. In *Y. lipolytica*, the corresponding reduction was 24%.

By combining the results of both models, we achieved the optimal composition of ISY medium. A statistically supported adjustment was made to enable the productive cultivation of auxotrophic *Y. lipolytica* strains, with concentrations of 10 $g*L^{-1}$ glucose and 2.29 $g*L^{-1}$ leucine to avoid limitations. For experiments with the double auxotrophic strain, uracil levels were increased to the same ratio as leucine (concentration = 0.456 $g*L^{-1}$). This ensured that neither of the additives, which are required due to auxotrophy, were able to limit growth.

3.4. Identification of growth-limiting media components

Media additives were classified into substance group clusters. The addition of FM22 trace element and vitamin solution had a

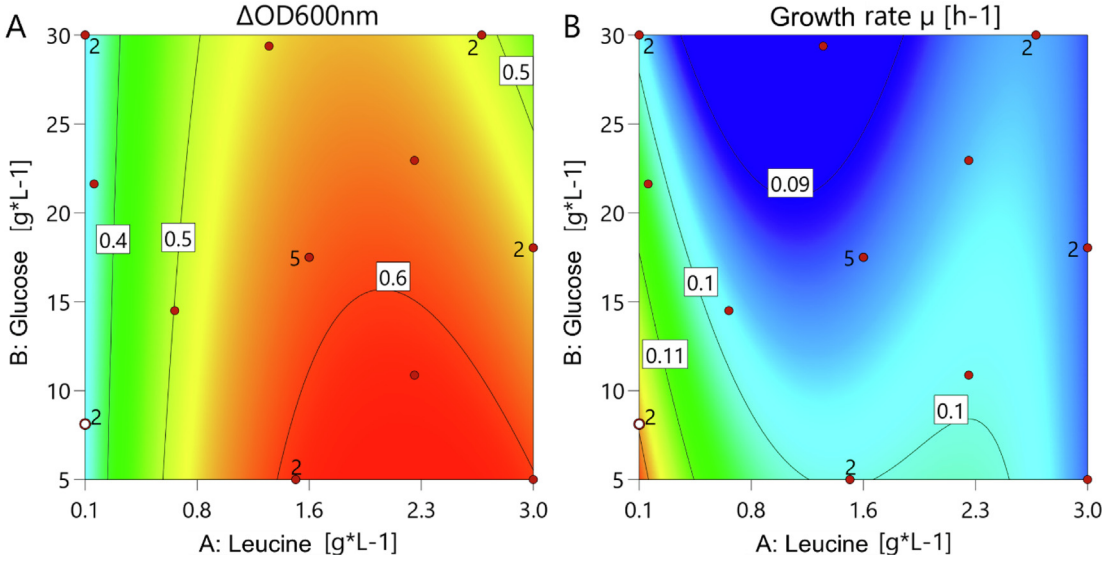

Fig. 1. Comparison of the effects of leucine and glucose concentrations on the growth of *Yarrowia lipolytica* PO1f, shown as a contour diagram. (A) Cell growth measured as ΔOD_{600nm} in the experimental period of 24 h. Model for growth: (ΔOD600nm)^2.67 = 0.036214 + 0.246449 A –0.000136 B – 0.001659 A * B – 0.054468 A^2. R^2 = 0.873. (B) Growth rate (μ) during exponential cell growth (7–14 h). Model for the growth rate: μ [h^{-1}] = 0.132482 – 0.052442 A – 0.001021 B + 0.000333 A * B + 0.030113 A^2 – 0.005675 A^3. R^2 = 0.862.

minor beneficial impact on cell growth, whereas doubling the salt content has had an adverse effect. A cluster of growth factors promoted a strong increase in cell growth, from which thiamine was identified as the only growth-limiting substance. By adding 0.2 mg^*L^{-1} thiamine hydrochloride, the ΔOD_{600nm} in shake flasks could be increased from 1.0 to 29.8. This effect was observed from a concentration of 0.02 mg^*L^{-1} up to the investigated upper limit of 2 mg^*L^{-1} without any significant difference in the biomass yield from 10 g^*L^{-1} glucose. Thiamine is required for the metabolism of amino acids and for the conversion of glucose to pyruvate [39]. This is confirmed by the significantly higher glucose consumption in cultures with thiamine compared to the observed reduced glucose consumption after 19 h of cultivation, in which traces of thiamine were carried over from the washed complex cryoculture and depletion occurred during cultivation. Higher concentrations of iron in the basic medium were tested in order to rule out possible limitations in high-cell-density cultivation due to insufficient freely available Fe^{2+}. Increasing the iron content to more than 120 mg^*L^{-1} Fe inhibited cell growth. In addition, there was a significant reduction in pH at higher iron concentrations. Based on a high basic value of 40 mg^*L^{-1} Fe and the limited buffer capacity of the ISY medium, higher concentrations cannot be tested in shake-flask cultures. To ensure a sufficient iron supply during cultivation, spiking experiments under controlled pH conditions are needed with sufficient oxygen input in the stirred-tank reactor.

The addition of supplements to batch bioreactor cultivations did not improve cell growth, confirming that the ISY medium contains sufficient iron, ammonium, thiamine and amino acids to metabolize 20 g^*L^{-1} glucose. Nevertheless, for biomasses exceeding 6 $g^*L^{-1,}$ the glucose consumption rate decreased drastically, indicating a further limitation. Given that ISY medium represents the elementary components of yeast cells, the ISY medium is not only suitable for cultivation in batch mode but also (and especially for) processes in fed-batch mode. For this purpose, a feed solution of ISY medium with the limiting component (glucose) present at a concentration of 100 g^*L^{-1} was used to demonstrate glucose-limited fed-batch cultivation. The limited growth rate of 0.200 h^{-1} during the fed-batch phase was verified by measuring the CDW, confirming a growth rate of 0.210 h^{-1}. No decline in glucose consumption was observed at biomasses greater than 6 g^*L^{-1}, underlining the suitability of the ISY medium for fed-batch cultivation.

In summary, a chemically defined medium based on the elementary composition of yeast was developed using statistical experimental designs and clustered supplement additions. It was suitable as a selective culture medium for auxotrophic *Yarrowia* strains. With the addition of thiamine hydrochloride and an optimized leucine and uracil concentration, the maximum cell density of the auxotrophic strain *Y. lipolytica* PO1f could be increased by a factor of 30. Furthermore, the successful cultivation of Y_LYs and *K. lactis* in the ISY medium highlights its universality. Enabling maximum specific growth rates of 0.305–0.352 h^{-1} and a yield of up to $Y_{x/s}$ = 0.56 g^*g^{-1}, this composition exceeds the rates of 0.16–0.18 h^{-1} and yields of $Y_{x/s}$ = 0.262 g^*g^{-1} reported for *Y. lipolytica* in other glucose-containing chemically defined media [10,40]. In addition to the universality and ability to select for auxotrophic strains, this medium is valuable for the development and efficient cultivation of Y_LYs and other *Yarrowia* strains.

CRediT authorship contribution statement

Oliver Birrenbach: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Peter Czermak:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Supplementary data

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Data availability

Data will be made available on request.

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