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## Methanol independent expression by Pichia pastoris employing derepression technologies

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**TITLE:**

Methanol Independent Expression by *Pichia pastoris* Employing De-repression Technologies

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**KEYWORDS:**

*Pichia pastoris*, *Komagataella phaffii*, promoter, derepression, feed control, shake flasks, cultivation

**SUMMARY:**

This method for the first time describes reliable experimental procedures for efficient inducible methanol-free recombinant protein production in *Pichia pastoris* (*Komagataella phaffii*), employing the carbon source regulated promoters  $P_{DC}$  and  $P_{DF}$  in small scale experiments while cultivation parameters can be monitored online, and a constant glycerol feed is applied.

**ABSTRACT:**

Methanol is a well-established carbon source and inducer for efficient protein production employing *Pichia pastoris* (*P. pastoris*) as a host on micro-, lab and industrial scale. However, due to its toxicity and flammability, there is a desire to avoid methanol while maintaining the high productivity of *P. pastoris*. Small scale bioreactor cultivations (0.5-5 L working volume) are commonly used to evaluate a strain and its protein production characteristics since microscale cultivation in deep well plates can be hardly controlled or relies on expensive equipment. Furthermore, traditional protocols for the cultivation and induction of *P. pastoris* were established for constitutive expression or methanol induction and so far, no reliable protocols were described to screen *P. pastoris* expression strains with derepressible promoters in (controlled and monitored) parallel cultivations. To simplify such initial cultivations to characterize and compare new protein production strains, we established a simple shake flask cultivation system for methanol free expression that simulates bioreactor conditions including a constant slow glycerol feed and online monitoring, thereby coming closer to the real conditions in bioreactors compared to mostly applied small scale batch cultivations. To drive recombinant protein expression in *P. pastoris*, the carbon source repressed promoters  $P_{DC}$  and  $P_{DF}$  were

applied. Polymer discs with embedded carbon source, releasing a constant amount of glycerol, assured a feed rate delivering the necessary energy for maintaining the promoters active while keeping the biomass generation low.

## INTRODUCTION:

Improvement in yield and reliable cultivation conditions on large scale for recombinant protein production by microorganisms such as bacteria or yeast is one of the major needs of today's biotechnology<sup>1</sup> and the commercial success of industrial applications. Due to simple cultivation procedures and media, high yields and well characterized tools<sup>2</sup> *Pichia pastoris* (*Komagataella phaffii*) is a widely used non-conventional yeast for recombinant protein production. Additional innovative tools are constantly developed for this species, like new expression vectors including new promoters as alternatives to the widely used  $P_{AOX1}$ , the promoter region of the alcohol oxidase 1 gene<sup>3-5</sup>. A 500 bp long DNA sequence upstream of the *CAT1* (*CTA1*) gene was identified as the promoter region, which enables a new methanol-free and versatile expression strategy in *P. pastoris*. The *CAT1* gene is the only catalase gene of *P. pastoris* and the protein is located in the peroxisomes. The catalase plays an important role in the detoxification of reactive oxygen species, which are arising, *e.g.*, from methanol oxidation in the peroxisomal MUT pathway or during beta oxidation of fatty acids<sup>6,7</sup>. The expression of the *CAT1* gene is repressed in the prescence of glucose or glycerol in the cultivation medium and derepressed upon the depletion of these carbon sources<sup>8</sup>. Moreover, the expression of the catalase gene can be induced with methanol and remarkably also with oleic acid, seemingly being the only gene of the *P. pastoris* MUT pathway that can be induced with oleic acid even reaching similar expression levels as with methanol induction<sup>9</sup>.

The 500 bp fragment of this *P. pastoris* *CAT1* promoter, which is called  $P_{DC}$  (sometimes also abbreviated  $P_{CAT1-500}$ ), has already been tested for the production of intracellularly expressed and secreted proteins and it proved to be an attractive alternative to the two classical *P. pastoris* promoters – the strong constitutive  $P_{GAP}$  and the methanol inducible  $P_{AOX1}$ , which were developed more than 20 years ago. The  $P_{DC}$  was able to reach 21% (CalB) and 35% (HRP) of the volumetric activities compared to the  $P_{GAP}$  in sugar rich medium. Upon methanol induction, the  $P_{DC}$  did not only respond faster than the  $P_{AOX1}$ , but could also clearly outperform it by a 2.8 fold higher final titer of CalB<sup>9</sup>.

Besides the  $P_{DC}$ , an orthologous promoter ( $P_{DF}$ ) had been identified exhibiting a similar regulation profile. However, it is significantly stronger than the  $P_{DC}$  under derepressed as well as under methanol-induced conditions (manuscript in preparation)<sup>3</sup>.

In cases where the strong constitutive  $P_{GAP}$  failed because of physiologically problematic or cytotoxic proteins<sup>10,11</sup>, the  $P_{DC}$  and the  $P_{DF}$  represent an ideal alternative. In micro scale experiments, the expression driven by these promoters starts after the depletion of the initial carbon source (*e.g.*, glucose or glycerol), which facilitates the biomass production without burdening the cells with the overexpression of the recombinant protein right from the beginning. While both of these promoters are still inducible by methanol, the activation by derepression makes additional use of the preinduction phase compared to the expressions employing  $P_{AOX1}$ .

Moreover, the  $P_{DC}$  and  $P_{DF}$  can be used for methanol-free protein production, which is a desirable goal for large industrial processes<sup>12</sup>.

For the development of applicable production strains and expression constructs, several steps in the development have to be passed in order to narrow down from large numbers of transformants to the preferred candidate for scale-up. Screenings usually are performed in high throughput in multi-well plates (micro scale: less than 0.5 mL) in order to capture a range of clones as large as possible. Subsequent re-screening and re-re-screening is the next step, followed by shake flask (small scale: 50-250 mL) or (micro-) bioreactor screenings<sup>12</sup>. However, it is desirable to have a method, which is most similar between the different scales from hundreds of microlitres in deep-well plates up to several milliliters in shake flasks up to later scale-up of methanol-free production in well-controlled bioreactors. Although shake flasks can provide already similar volumes as small bioreactors, there are several limitations, which are highly relevant for large scale protein production such as constant feeding, aeration and online-monitoring<sup>13</sup> of growth and oxygen supply. Optical monitoring employing adapted shake flasks and shaking devices provide a cost-effective alternative to more sophisticated equipment for parallel cultivation while still providing constant online information about major culture parameters such as biomass and dissolved oxygen concentration. Slow release of carbon source from polymer carriers can be applied to secure a constant and controlled feed without relying on complex technical solutions and devices, simulating more similar conditions of bioreactors than the previously applied simple full depletion of carbon source<sup>9,10</sup>, where energy for ongoing protein expression becomes limiting.

The following method describes a small to medium scale methanol-free controllable protein expression system in *P. pastoris* based on the new promoters  $P_{DC}$  and  $P_{DF}$ . Induction by derepression involves two phases. A first short phase of biomass accumulation without the production of the protein of interest, using a carbon source that represses the promoters and a second phase of target protein production, where a carbon source is supplied in small but constant concentrations maintaining the promoter derepressed. Online monitoring of most critical cultivation parameters is applied during the whole cultivation period. The goal was to provide a methanol free, reliable and scalable cultivation system for *P. pastoris*.

## **PROTOCOL:**

### **1. Media Preparation**

1.1. Buffered minimal media (BMG with glycerol, BMD with dextrose as carbon source) preparation (1 L)

1.1.1. Autoclave 650 mL of distilled water in a 1 L screw cap glass bottle. After cooling down, complete the medium by adding 100 mL of autoclaved 10x YNB (134 g/L yeast nitrogen base w/o amino acids), 200 mL of potassium phosphate buffer (1 M, pH 6), 30-100 mL of 10% w/v glucose or glycerol (depending on the desired generated biomass amount during the batch), 2 mL of sterile 500x Biotin (10 mg/mL) solution and fill up to 1 L with sterile distilled water.

Note: All ingredients have to be autoclaved in separate flasks. Biotin is destroyed when autoclaved, and therefore has to be filter sterilized with 0.2 µm membrane filters.

## 1.2. Buffered rich media (BYPG) (1 L)

1.2.1. Autoclave 700 mL of distilled water with 1% w/v yeast extract and 2% w/v peptone in a 1 L screw cap bottle. After cooling down, add 30-100 mL of the separately autoclaved 10% w/v glycerol, 200 mL of potassium phosphate buffer (1 M, pH 6) and fill up to 1 L with sterile distilled water.

## 2. Shake Flasks Preparation

2.1. Add 5 mL of distilled water in the 250 mL baffled shake flasks, cover it with two layers of cotton cloth and fix it in place with rubber bands. Cover the cloth with a piece of aluminum foil.

2.2. Autoclave the flasks at 121 °C for 20 min for full sterilization.

2.3. Remove residual water from the flask and aliquot 50 mL of the previously prepared media to each flask.

## 3. Overnight Culture Inoculation

3.1. Start an overnight culture (ONC) with a single fresh colony of the desired expression strain in 5 mL of YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) in a 50 mL centrifuge tube while leaving the lid slightly open to allow proper aeration.

3.2. Let it grow over night at 28 °C, 80% humidity, in a shaker at 100-130 rpm (2.5 cm shaking diameter).

## 4. Measurement Setup and Main Culture Cultivation

4.1. For setting up the online monitoring system, follow the manufacturer's instructions for appropriate calibration of the devices.

4.2. Place a computer, where the appropriate software for monitoring is installed in range for a Bluetooth connection to the measurement stations. Start the software to connect the devices via Bluetooth.

4.2.1. Turn on the Bluetooth connection on the biomass measuring device by pressing the silver button on the front.

4.2.2. In the software, click **Devices** then **Find Devices**.

Note: The devices should appear as a list in the window below the task line. If they do not, it is advisable to check if the batteries are charged and the computer is within reach for a Bluetooth connection.

4.2.3. Drag and drop the found devices to the squares on the right side of the screen **Measurement Tray**.

4.2.4. Click **Connect**.

Note: When the connection was successful, a control screen appears.

4.2.5. To start a test run for the Bluetooth connection, click **Measurement**.

4.2.6. Set up the experiment.

4.2.6.1. Click **Start Measurement**, name the experiment and enable all parameters needed to monitor.

Note: Licenses are needed for each of the possible measured parameters.

4.2.6.2. Set **Interval** to 3 min.

Note: The interval determines how often measurement points are taken and every measurement point results in a value afterwards. Measuring every minute is very accurate, but lots of data are generated which makes it more laborious to evaluate.

4.2.6.3. Set **Average Measurement Points** to 11.

Note: This setting determines how many measurement points are actually taken every 3 min. Therefore, the output is the mean value of 11 measurement points over 11 s.

4.2.6.4. Enter the names for the samples.

4.2.6.5. Skip the next screen, as the angle was already calibrated before (Step 4.1).

Note: A test run for the Bluetooth connection before inoculating the main cultures is advised to ensure stable connections and appropriate location of the connected computer. Also, the batteries have to be charged.

4.3. Measure the cell density of the ONC (Step 3.1) diluted in cultivation media (1:20) in a spectrophotometer at 600 nm wavelength. Inoculate 50 mL of the medium (recommended carbon source concentration 0.3-1%, different media possible as described in Step 1.) with the ONC to an OD<sub>600</sub> of 0.05 by using the following calculation.

$$\frac{50 \text{ mL} * 0.05}{OD_{600} (ONC) - 0.05}$$

4.4. Place the flasks in the detectors and shake at 28 °C, 130 rpm and 80% humidity.

Note: It is very important to give the culture 5 min of shaking before starting the measurement to avoid cells sticking to the bottom of the flask and yielding wrong values.

4.5. Click **Start Measurement** in the software.

4.6. Take 0.2 mL samples for cell density measurement 4 h after the inoculation and when the carbon source gets depleted (determination described in step 4.7) in triplicates. Dilute the samples appropriately in cultivation media (first measurement 1:5, second measurement 1:20) and measure the absorption at 600 nm wavelength in a spectrophotometer. Before removing the flasks and even before stopping the shaker, always pause the measurement in the software.

Note: The detectors are calibrated for recording the parameters under defined shaking conditions and will yield to nonsense measurements when recording still cultures. Also, make sure to always wait 5 min of shaking before starting the measurement again. The cell density measurements are essential because the detector is delivering just values for the biomass and not real cell density. A calibration function between the OD<sub>600</sub> values (x values) and the values obtained by the online measurement system (y values) can be achieved by measuring several timepoints spectrophotometrically. The values are not in a linear but in a logarithmic relation according to the following calibration function:

$$y = k * \ln(x) + d$$

y: OD<sub>600</sub> values obtained by spectrophotometer

x: values obtained by online measurement system

k: slope

d: axis intercept

The slope and the axis intercept can then be used to convert any value into the corresponding OD<sub>600</sub> value. In Excel one can plot the spectrophotometrically obtained OD<sub>600</sub> values against the values from the online system from the same timepoint. The addition of a logarithmic trendline and the corresponding equation will give the calibration function shown above.

4.7. Cultivate until the carbon source is nearly depleted (app. 12-20 h). For that purpose, let the cultures grow until it can be seen in the software diagrams that the oxygen concentration is approaching zero and the cells are in the exponential growth phase.

Note: This is the case for carbon source concentrations used in this protocol. The timepoint of carbon source depletion can be determined with the online monitoring system as it is shown exemplarily in **Figure 1** in the Representative Results section, when the oxygen level approaches zero and the cells are in the exponential growth phase.

## 5. Protein Expression by De-repression

5.1. When the timepoint described in Step 4.6 is reached, start feeding the cultures by the addition of four feed discs per flask under sterile conditions.

Note: Under the described conditions, four glycerol feed discs release 2 mg/h glycerol according to the manufacturer.

5.2. Continue the cultivation for 60-90 h while taking samples every 24 h to monitor the protein expression by an assay of choice (e.g., Bradford assay<sup>15</sup>, SDS PAGE<sup>16</sup> or activity assays) and cell density measurements.

## 6. Culture Harvesting

6.1. After the 60-90 h of cultivation, fill the cultures in 50 mL centrifuge tubes for harvesting by centrifugation at 4000 x g, 4 °C for 10 min.

Note: The supernatant (for secreted proteins) or the cells (for intracellular protein expression) can be harvested by centrifugation at 4000 x g, 4 °C for 10 min and further analyses can be done.

6.2. Export the online measurement data as a spreadsheet file from the software for further analysis.

6.3. Fill 5-10 mL of distilled water to all the flasks and autoclave to inactivate the remaining cells.

## REPRESENTATIVE RESULTS:

As described in the protocol section, the software records the important cultivation parameters over the whole cultivation. **Figure 1** shows the visualization of biomass development and oxygen concentration during a cultivation started with either 0.5 % (**Figure 1A**) or 0.3 % (**Figure 1B**) carbon source in the batch followed by the addition of glycerol feed discs. The online monitoring system is especially useful for the de-repressed cultivations as the timepoint of carbon source depletion after the batch can be determined exactly. As it can be seen in **Figure 1**, the oxygen concentration in the medium is decreasing rapidly and approaching zero, especially for 0.5 % glycerol in the batch (**Figure 1A**) while the biomass is increasing exponentially at this point. Here, the cells are in the exponential growth phase and in order to make optimal use of the carbon source release, shortly before the culture reaches the stationary phase, the feed discs should be added as it is described in the protocol. The addition of the feed discs before the oxygen concentration reaches zero is important for de-repressed protein expression to prevent the cells from oxygen limitation. Lower glycerol concentrations reduce the effect of short-term oxygen limitations as it can be seen in **Figure 1B** and are therefore recommended for de-repressed protein expression.

In the following, the results of two different cultivation experiments – employing the described protocol and the monitoring system shown in **Figure 1** – are described. In the first experiment, a *P. pastoris* strain producing the glucose oxidase from *Aspergillus niger* (*A. niger*) under the control of the  $P_{DC}$  and in the second a *P. pastoris* strain producing the human growth hormone under control of the  $P_{DF}$  is shown.



In this experiment, glucose oxidase production under the control of  $P_{DC}$  was investigated. Therefore, different cultivation strategies were compared: glycerol pulsing, constant glycerol feed by addition of feed discs and cultivation without any feed or pulsing. The cultivation was done in 50 mL buffered minimal media with 0.5% glucose as sole carbon source in 250 mL shake flasks (**Table 1**).

Upon 160 h cultivation, the highest biomass concentrations were obtained, when a feeding strategy using a glucose batch for biomass production (38 h) and glycerol pulsing (0.25% w/v glycerol after 38 h, 61 h and 86 h cultivation) was employed (0.21 g/L total secretory protein, 8.4 U/mL). Although a 2-fold reduction of the biomass concentration and the total amount of secreted protein were obtained, when the feed discs were added after 38 h glucose batch, the volumetric activity was even 1 U/mL higher compared to the cultivation using glycerol pulsing. This indicates that a substantial amount of secreted protein does not contribute to the volumetric activity in the supernatant and may be secreted in an inactive form. Therefore, higher glycerol concentrations from glycerol pulses seemingly favor the formation of biomass instead of target protein production and constant low glycerol release led to more than two-fold higher specific productivity.

Another experiment employing the de-repressed expression method was done by expressing the human growth hormone (hGH) under control of  $P_{DF}$ . The construct was stably integrated into the genome of *P. pastoris* strain BSYBG11. Here, 50 mL of BYPG, buffered rich medium, in 250 mL baffled shake flasks was used with 0.3% (w/v) glycerol as carbon source in the batch. Upon glycerol depletion, the protein expression with constant glycerol feed was ensured by the addition of 4 feed discs per flask and compared to no glycerol feed (**Figure 2**).

**Figure 2** shows the comparison between hGH expression and biomass development of the same strain cultivated with and without constant glycerol feed. As assay sandwich ELISA was used with the secondary antibody fused to HRP and detection was carried out with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. Especially for this protein, it seemed to be the case that without any feeding, the cells start to degrade the produced protein after approximately 30 h (light blue squares), whereas by feeding, this degradation could be prevented (dark blue triangles) and even the protein concentration per biomass still increased after 150 h of cultivation. As already observed for *Hansenula polymorpha*<sup>14</sup>, this result shows how switching from batch, like it is usually used in shake flask scale, to fed-batch mode can optimize *P. pastoris* cultivations.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Visualization of online recorded parameters during two cultivations with different glycerol concentrations in the batch.** The cultivations were started with 0.5% w/v (**A**) and 0.3% w/v (**B**) glycerol in the batch followed by the de-repression phase where the cells were fed by applying 4 feed discs per flask (release rate of 0.5 mg/h/disc according to the manufacturer). The dashed lines show the oxygen concentration in the medium, whereas the continuous lines show the cell density (OD<sub>600</sub>). Error bars show the standard deviation of six replicates (2 biological, three technical each).

**Figure 2: Comparison of human growth hormone (hGH) expression in batch and fed-batch**

**mode.** hGH content is indicated by absorption values at 405 nm by an ELISA where the 2<sup>nd</sup> antibody was an HRP fusion and TMB was used as a substrate for detection. Shown are the results for two different cultures: light blue line and squares display the cell density and hGH concentration normalized by the cell densities of a culture without any feed after the batch, compared to a culture, where the glycerol feed discs were applied (dark blue lines and triangles). Error bars show the standard deviation of six replicates (2 biological, three technical each).

**Table 1: Results of methanol independent GOX expression under the control of the de-repressed promoter  $P_{DC}$ .**

## **DISCUSSION:**

This method presents a reliable protocol for efficient methanol independent inducible expression by *P. pastoris* as an alternative to classical methanol induced protein expression driven by  $P_{AOX1}$ <sup>12</sup>. De-repressible promoters, like the  $P_{DC}$ , the  $P_{DF}$  or previously described mutated  $P_{AOX1}$  variants<sup>17</sup>, build the molecular basis for this new protein production concept. The shown representative results underline the usefulness and practicability of recombinant protein expression in *P. pastoris* by de-repressed promoters and small-scale screening with simple online monitoring. On the one hand, the harmful and toxic methanol that is usually used for the induction of the  $AOX1$  promoter can be eliminated from the cultivation. On the other hand, the growth and the protein production phase are uncoupled<sup>9</sup> in contrast to constitutive protein expression, *e.g.*, with the commonly used  $P_{GAP}$ . This is especially beneficial when the cells should express toxic or harmful proteins that display a burden for them.

The possibility to start the cultivations with different carbon source concentrations enables the decision of how much biomass one would like to gain before the protein production phase starts. When choosing a lower carbon source concentration in the batch, oxygen transfer limitations due to too high cell densities can be reduced while on the other hand higher cell densities can also result in higher protein titers. By monitoring the culture development in terms of biomass generation and oxygen concentrations, the optimal time-point for fed-batch initiation can be determined easily as it is shown in **Figure 1**.

For online monitoring of these cultivation parameters, an appropriate measurement system should be applied. The biomass measuring device (*e.g.*, SFR Vario) is a simple and economic online measurement system for culture monitoring in shake flasks. The optoelectronic reader determines cell growth in the flask via scattered light detection and reads out the signal of the oxygen sensor integrated in the flask. The optics for biomass measurement consist of an LED that emits a light signal, which is scattered by particles (cells) in the culture broth and detected with a photodiode. The optical sensors emit fluorescence when excited with light of a certain wavelength. Depending on the amount of analyte molecules present, this fluorescence signal changes, which is detected by the reader optics and translated into concentration values. However, a cell density measurement calibration has to be established beforehand, since the measurement system is not measuring actual OD<sub>600</sub> values. The oxygen uptake rate can be calculated from the slope of the oxygen curve with the reader software, and the temperature as well as rpm are recorded continuously together with the other parameters. To enable monitoring

with this system, the shake flasks have to be previously equipped with the appropriate sensors for the desired parameters.

By the addition of four slow-release polymer discs providing a constant amount of glycerol to the culture broth, biomass accumulation is nearly stopped, and recombinant protein production is continued. The determination of the feed disc addition time point is not a critical step in this experiment, but it should be considered that a premature feed start before the exponential growth phase could be inhibiting the promoter activation as the expression starts upon carbon source depletion. Additionally, the total feeding time due to polymer capacity limitations may be reduced, whereas delaying the feed after the cells have reached the stationary phase might lead to starving and degradation of already produced protein. The amount of feed discs used in this protocol was determined experimentally for the optimal carbon source release to keep the promoter de-repressed while holding biomass generation low (data not shown). In this manner, the cultivations can be performed easily over 160-180 h without any intervention.

Main applications of this new protocol will be in expression clone screening, enzyme evolution and the discovery, the characterization and engineering of new promoters employing a methanol-free cultivation protocol in well-monitored conditions. In principle, the same protocol should be applicable for mixed-feed strategies employing methanol and limited fermentative carbon source feeds such as described by Panula-Perälä *et al.* in 2014<sup>18</sup>.

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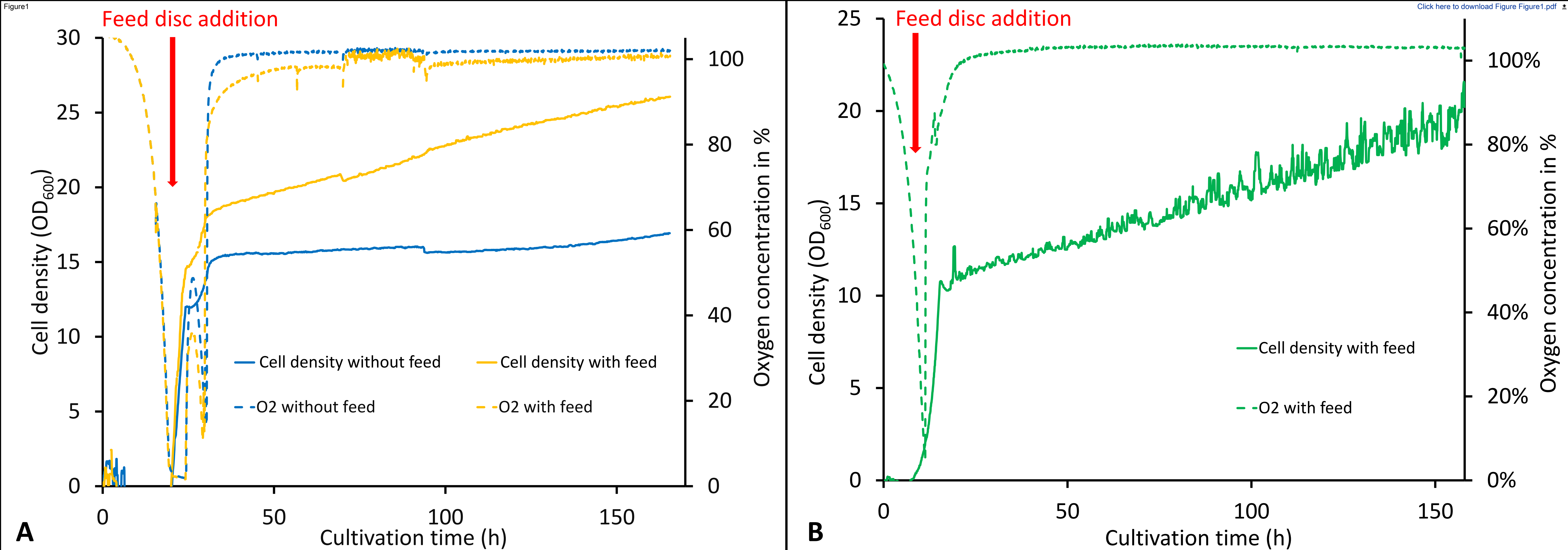
#### DISCLOSURES:

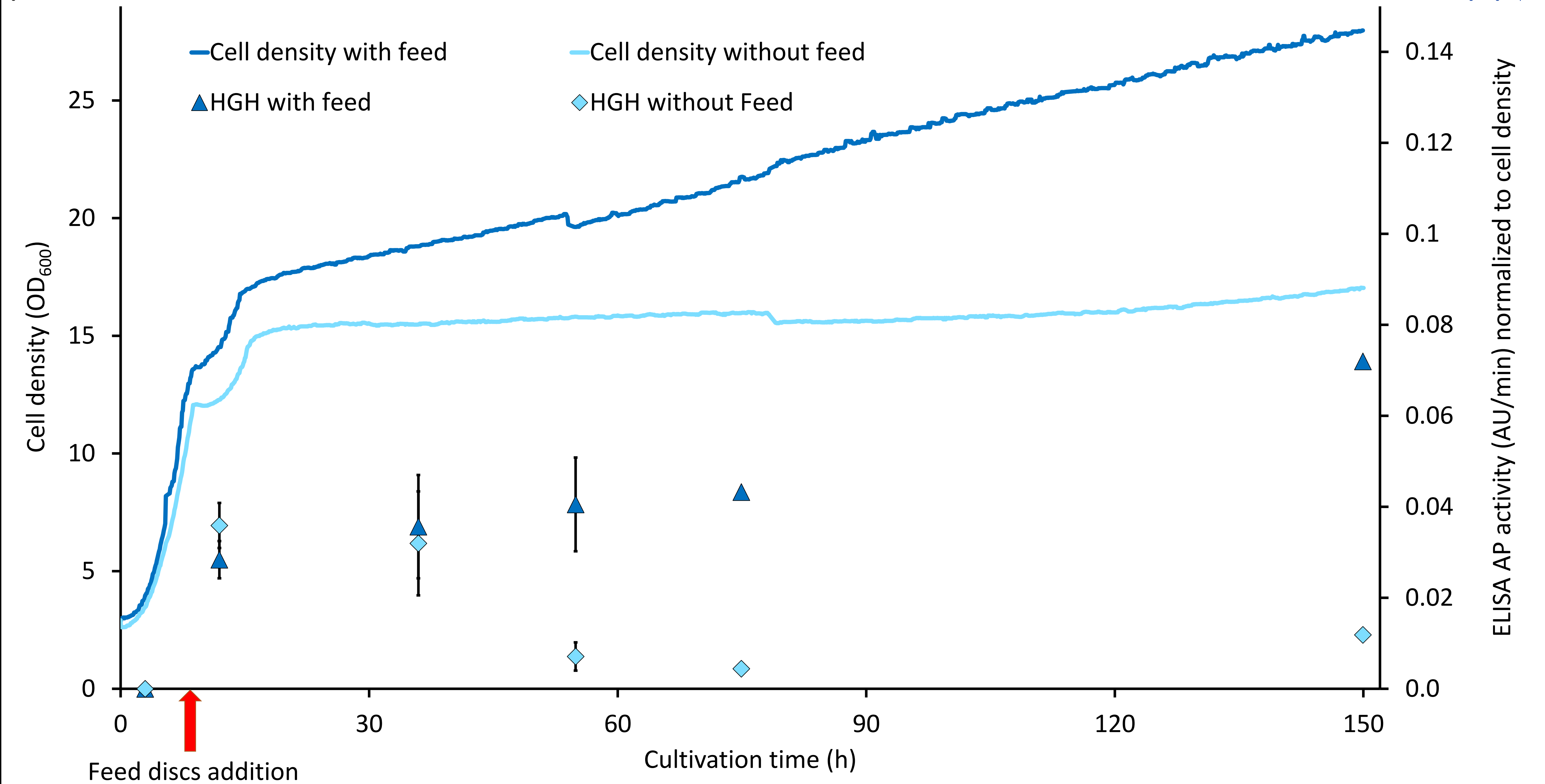
Although the presented protocol is generally applicable for cultivation and induction under derepressed conditions, bisy e.U. declares to have an interest in commercialization of the new derepressed promoters used in this study.


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Figure2.svg



P <sub>DC</sub> -GOX at 160 h cultivation time	OD <sub>600</sub>		vol. activity [U/mL]		total amount of secreted protein [mg/mL]	
	Mean	SD	Mean	SD	Mean	SD
BMD1% batch + glycerol pulsing	74.6	2	8.4	2.4	0.21	0.01
BMD1% batch + constant glycerol feed	32.5	0.3	9.4	0.7	0.09	0.01
BMD1% batch	18.8	0.6	3.7	0.7	0.01	0

<b>volumetric activity per cell density</b>
Mean
0.11
0.29
0.2

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Bacto Yeast Extract	BD Biosciences	212720	
Baffled Flask 250 mL	DWK Life Science	212833655	
BBL Phytone Peptone	BD Biosciences	298147	
BioPhotometer	Eppendorf AG	5500-200-10	
Certoclav-EL	CertoClav GmbH	9.842 014	
Difco Yeast nitrogen base w/o amino acids	BD Biosciences	291920	
Feed discs glycerol	Adolf Kuhner AG	315060	
	Carl Roth GmbH + Co.		
Glucose-monohydrate	KG	6887	
	Carl Roth GmbH + Co.		
Glycerol ≥98 %	KG	3783	
	Carl Roth GmbH + Co.		
K <sub>2</sub> HPO <sub>4</sub>	KG	6875	
	Carl Roth GmbH + Co.		
KH <sub>2</sub> PO <sub>4</sub>	KG	3904	
Multitron Standard	Infors AG	444-4226	
SFR Vario v2	PreSens GmbH	200001689	



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Dear Reviewer,

Thank you for your revision. We did not want to compare shake flask cultivations to bioreactor cultivations and achieve the same protein titers in shake flasks. Expression clones that may perform well in a micro-scale screening where constant feed is hardly possible and parameters such as cell density and oxygen are usually not monitored in high-throughput, might yield totally different results in following bioreactor cultivations where all parameters are defined and controlled. This cultivation method which was especially developed for the described derepressed promoters displays a compromise between impossible control of parameters and throughput amount. Experiments in our lab showed in the past that the performance of respective clones (in relative comparison to each other) in shake flasks using this cultivation method is much more applicable to their later performance in bioreactors than batch or pulsed cultivations in shake flask scale as in this method also a limited feed strategy is applied by the slow carbon source release. For this kind of derepressed protein expression a good monitoring system even in shake flask scale is extremely useful to determine the optimal timepoint for fed-batch start. Of course, this cultivation method is not replacing bioreactor experiments and also derepressed protein expression with the mentioned promoters yields higher protein titers in bioreactors, but by employing this method one can limit the number of possible candidates for laborious large-scale cultivations by preselecting clones that perform well under simulated bioreactor-like conditions due to the more similar behavior of the strains compared to batch or pulsed feed cultivations.

Dear Reviewer,

Thank you for your revision. Our method is about methanol-independent expression.

The described method by Panula-Perälä et al. (2014) describes a way to yield higher protein titers by a slow release of glucose and methanol induction at the same time. However, our method is exclusively for methanol-independent protein production, which is from great interest, especially for large-scale cultivations, where this toxic and flammable inducer should be avoided. Therefore, comparing this expression strategy to the AOX1 promoter makes no sense to us, since methanol induction must be applied to achieve any protein production at all, which is not the aim of our method. Nevertheless, we included the recommended reference in order to show that similar procedures might be feasible with glycerol release.



Dear Editor,

Please find the performed changes in the manuscript below.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Proofreading was done.*

2. Please do not highlight notes for filming. In order to film a note, please write it as a step in imperative tense.

*The notes were spared from highlighting.*

3. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

4. Step 4.3 Does the preculture refer to the culture in step 3.2. Please specify.

*Yes it does. It was specified: "4.3. Measure the cell density of the ONC (3.1) diluted in cultivation media 1:20 in a spectrophotometer at 600 nm wavelength."*

5. 4.5: How to set up the Bluetooth connection?

*The Bluetooth set-up was described in detail:*

*4.2. Place a computer, where the appropriate software for monitoring is installed in range for a Bluetooth connection to the measurement stations. Start the software to connect the devices via Bluetooth.*

*4.2.1. Turn on the Bluetooth connection on the SFR Vario devices by pressing the silver button on the front.*

*4.2.2. In the software, click "Devices" then below "Find Devices".*

*Note: The devices should appear as a list in the window below the task line. If they don't it is advisable to check if the batteries are charged and the computer is within reach for a Bluetooth connection.*

*4.2.3. Drag and drop the found devices to the squares on the right side of the screen "Measurement Tray".*

*4.2.4. Click "Connect".*

*Note: When the connection was successful a control screen appears.*

*4.2.5. To start a test run for the Bluetooth connection click "Measurement".*

*4.2.6. Set up your experiment.*

*4.2.6.1. Click "Start Measurement", name your experiment and enable all parameters you want to monitor.*

*Note: Licenses are needed for each of the possible measured parameters.*

*4.2.6.2. Set "Interval" to 3 minutes.*

*Note: The interval determines how often measurement points are taken and every measurement point results in a value afterwards. Measuring every minute is very accurate but lots of data are generated which makes it more laborious to evaluate.*

*4.2.6.3. Set "Average Measurement Points" to 11.*

*Note: This setting determines how many measurement points are actually taken every 3 minutes. Therefore, the output is the mean value of 11 measurement points over 11 seconds.*

*4.2.6.4. Enter the names for your samples.*

*4.2.6.5. Skip the next screen, as the angle was already calibrated before (4.1).*

6. 4.6: How to take samples? Dilute the sample with what?

*The sample volume was specified, as well as the dilution procedure:*

*4.6. Take 0.2 mL samples for cell density measurement 4 h after inoculation and when the carbon source gets depleted (determination described in 4.7) in triplicates. Dilute the samples appropriately in cultivation media (first measurement 1:5, second measurement 1:20) and measure the absorption at 600 nm wavelength in a spectrophotometer. Before removing the flasks and even before stopping the shaker always pause the measurement in the software.*

7. 4.7: How to determine if the carbon source is depleted?

*The details for carbon source depletion determination were moved from the note below into 4.7:*

*4.7. Cultivate until the carbon source is nearly depleted (app. 12-20 h). For that purpose, let the cultures grow until it can be seen in the software diagrams that the oxygen concentration is approaching zero and the cells are in the exponential growth phase.*

8. Please use h, min, s for time units.

9. Please remove all headers from Representative Results.

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*Points 8-11: Changes were included as advised.*