Turbidity of *Saccharomyces cerevisiae*: a proposed cell quantification method

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Abstract. The relation between cell and turbidity (cloudiness) in suspended form is studied intensively to facilitate the estimation of the cell number. A standard curve in turbidimetry has utility to compare the turbidity reading value for the determination of *Saccharomyces cerevisiae* cell number and using a model. A standard curve is a prospect in this issue for the cell that has an inherent inaccuracy in the method as well as spectrophotometry. This research is conducted to obtain a model for the standard curve using analysis and diagnosis of the linear regression which was measured the relative standard error (SE) value of the dependent variable by simulation of turbidity value and verification of the applied model. We obtained the smallest relative SE from one of the five datasets that resulted in less than 10% of the relative SE on the estimated cell number. Plotting all of the bivariate data resulted in a larger error on the scattered light intensity at the higher cell number value, and this characteristic was named heteroscedasticity. Application of turbidity method for cell enumeration per mL in the media was significant equal and lower coefficient variation than the result of verification by a viable method.

1. Introduction

The interaction between light spectrum of 600 nm and a yeast cell is a relation that has been studied using the spectrophotometry method for the estimation of the *S. cerevisiae* cells quantity. However, it was deemed not appropriate [1], photometer technique on McFarland method has one significant difference of two mean values that was analyzed by coefficient of variation (CV) toward bacteria cells [2] and low accuracy by standard deviation [3]. The cell is a live particle and it is not dissolve in water but suspended form. The aqueous suspended solid, water-insoluble particles were passed through the light spectrum and the absorbance, transmittance, and scattered light occurring together.

Direct method by microscopic techniques allows our eyes to see the particles of cells that dilute previously and counting. The bigger cell like yeast in haemocytometer is utilized conventionally under microscope to quantify the cell concentration per mL as direct method [4]. The form of suspended yeast cell result turbidity and is interacted with light in turbidimeter as a new turbidity techniques. The turbidity method (turbidimetry) uses curve as standard comparator for a turbidity value to determination of the cell number per mL.
The biological particles of *Scenedesmus sp*, *Chlamydomonas sp*, and *Chlorella sp* have a linear pattern on the quantity of the $5 \times 10^4 - 1 \times 10^6$ cells interval and uses 633 and 690 nm of light spectrums [5]. The utilization of that wave length is important in relation with the particles in suspended solid. The light scattering causes attenuation in suspended cells as the particles reflect incident light randomly and in the instrument that catches the attenuation light at a 90° angle towards the incident light – which is suggested by the Mie theory application of light scattering. The analogy is that the particles in the form of suspended solid, air, or aerosols [6] and soil particles [7]. The characteristic of scattered light in the turbidimetry method can be used to estimation the quantity of the cells by a standard curve; a regression equation. Portable instruments are currently available for the suspended particles but there is no significance, inherent inaccuracy, and no precision model on cell number estimation scope.

An incident light has three possibilities in the aqueous suspended solid that can be transmitted, absorbed, and scattered by the dispersant or solvent, coloured things, and particles. That transmitted and absorbed light is explained by the photon character of light. The scattered light by the particles in suspended solid is another character of light; the reflected spectrums are electromagnetic waves towards size and number of particles. If the particles surface is not smooth or rough like the *S. cerevisiae*, the spectrum will be reflected in random directions as diffusion reflections and will cause it to be brighter in suspended solid of liquid forms.

The incident light has a lot of reflected light as compared to refracted light in liquid form. Although there are a few pigments on surface of particles that absorb the energy of spectrum, the width of the surface that reflects the light is great. A pigment named melanin which is not represented by the *S. cerevisiae* [8]. The cell wall of the fungi has a pigment in relation to the pathogen function [9]. The melanin pigment absorbs ultra violet spectrum that is less than 400 nm wavelength.

The interaction between light and the biological cell has many characteristic lights such as reflection that consist of surface and subsurface scattering, refraction in epidermis cell of human skin, total absorption in papillary dermis by the β-carotene-oxyhaemoglobin-deoxyhaemoglobin-bilirubin, and propagation trough the layer of skin cell using 400 – 700 nm of spectrum [10]. Research showed that the reflectance was higher in lightly pigmented skin specimen as compared to moderately pigmented specimen. These mean that in fewer pigmented skin cells, many reflectance occur (scattering).

Turbidimetry is a method of measuring light scattering by particles and it has a linear relationship on total suspend solid (TSS) in sewer systems [11]. Increasing scattered light as the concentration of particles increases and causes multiple scattering and the absorption of light to increase [12]. Decreasing scattered light could be used for dispersant and physical treatment which can decrease the aggregate [13].

The linear relationship was translated to the regression model and four stages was identified which included identification, estimation toward the parameter of model, testing or diagnostic checking, and implementation or applications [14]. The stage of diagnostic checking was aimed to test whether the model had a significant regression relationship between the variables. A system of diagnostic is needed to determine how each stage was structured about estimated models.

A mathematic model is a form of equation that consists of variables and states the relation among them. Model is a functional relationship among variables and the one model tool that can be used is regression. The significance for this relation in spectrophotometry is definite regression that would be measured by coefficient of regression determination ($r^2$), the accuracy by standard error (SE), and the precision by deviation standard [15]. The aim of this research is to obtain a relation model that consists of the independent variable of cell number in the dispersant and the dependent variable of turbidity by analysis and diagnosis of linear regression.
2. Material and Methods

2.1. Materials

Tube centrifugation with PVC cover, tissue paper, Ethanol swab tissue, buffer pH 6.8; pH 7, pure aquadest (OTSUKA) 25 mL, tab water, surfactant cleaner, shelf test tube, turbidity standard of 10, 40 dan 100 NTU (Sigma Aldrich), granules of Saccharomyces cerevisiae product (Sigma Aldrich), Disodium Hydrogen Phosphate anhydrate (Merck), Sodium dihydrogen Phosphate dihydrate (Merck), Chloride Acid (Merck), Absolute Ethanol (Merck), Sodium Chloride (Merck), potato dextrose broth, Micro pipet 1000-5000 μL (D-LAB) and accessories. OrbecoHellige digital-direct reading model 965-10A Turbidimeter using Tungsten lamp and accessories, voltage stabilizer, Denver Instrument Company AA160 balance, Neubauer (Improve/Tuerk) type of Haemocytometer, Binocular microscope-Personal Computer with Windows system-CCTV-stabilizer voltage, stick pH-meter, refrigerator. Standardize instrument of turbidimeter is as follows: the use of 0 – 20.00 scale was standardized with turbidity of 10 NTU, 0 – 200 scale with 40 NTU, and 0 – 1000 with 100 NTU, Rotary shaker VRN-200, Autoclave, pH meter Hanna Instrument HI8424, Condenser and rubber.

2.2. Searching model: standard curve

The experiment method used regression design [16] on two paired variables (bivariate) according to the linear relationship [11]. The independent variable was the cell population (X) of Saccharomyces cerevisiae in the suspended cells form per volume (mL). The dependent variable was the turbidity (Y). Bivariate size was 10 (X; Yi) as a data set and was replicated five times [17]. The evidence of characteristic heterochedasticity [18] is the pattern of the all data of NTU that plotted for graph [19].

The experiment, analysis and curve diagnose were done as follows: First, the S. cerevisiae cell was suspended in Ethanol Saline pH 4.9 Phosphate Buffer [13] (ES-4.9PB) and prepared using haemocytometer; secondly, dependent variable was the turbidity of suspended cells. Normality of paired data was analysed using the standardized residual of bivariate. The test of backward-Cook’s D and centred Leverage used Cook’s D value as variable of Y and centred Leverage value using variable of X to plot the relation [19]. The possible elimination of a paired datum, one bivariate, showed a much distorted dot in the plot. The dot was eliminated and resulted in a new regression equation. Eliminations of a dot were measured using the variance (Vx) value [20-21] of predicted cells by the regression equation.

The procedure for aggregate reduction of the cell was done as follows: Phosphate saline buffer was made using 125 mL of Disodium hydrogen phosphate and adding 125 mL Sodium di-hydrogen Phosphate to respective 0.05 M [13]. Adding HCl 10% to pH 4.9; Sodium Chloride to 0.095% and Ethanol absolute was added to 10% (w/v). This formula was termed ES-4.9PB. Granules of S. cerevisiae was added with ES-4.9PB as suspension and was shaken by hand six times, and incubated for 30 minutes. The suspended solid was centrifuged at 8000 g in two stages of 2 x 10 minutes.

Quantity of cells (X) was calculated at low concentration in haemocytometer using grid 1 mm² area in 4x4 boxes and thickness of 0.1 mm. High concentration of cell using 1 mm² area in the center of 5x5 boxes [22]. The quantity of cells was calculated to average in volume of 1 cm³ or mL by conversion value. Reduced quantity of suspended cell in the bottle of turbidimeter was done as follows by filling 15 mL of pure water and adding high suspended cells, shaking the bottle, and utilizing the drop pipet to calculate the cells in the haemocytometer. The turbidity values (NTU) of suspended cells were obtained from respective values of those cell quantities.

2.3. Applying and testing standard curve

The experiment used design of t-Test from two population means; n1 and n2 more than 10 data. The comparator is plate agar technique and still debate but the consideration was only one species of cells, dilution, and relative standard deviation [23].

The sample of plate agar technique was taken from fermenters; the random result of cells incubation more than three hours; a mL of suspended cell from a V mL of fermenter was added 10 mL with sterile
distilled water (Figure 1), shaken until homogenous suspension. It was taken 0.1 mL of suspended cell and added to 20 mL sterile distilled water; 0.1 mL for inoculation to plate of potato dextrose agar (PDA). It was incubated in 30 °C for 24 – 48 hours and was counted the colony forming unit (CFU). Furthermore, number of CFU and the diluted factor (P) to count Σ cell/mL.

\[
\Sigma \text{cell/mL} = \left[ \left( \frac{1}{a} \right) \times 2.0301 \times 10^4 \right] \times P
\] (1)

The diluted factor depend lower or higher cell concentration in the fermenter respectively.

\[
\Sigma \text{cell/mL} = \left[ \frac{\text{NTU} - 2.955}{1.47} \right] \times 10^5 \times \frac{10^V}{b} \times P
\] (2)

Statistical testing toward two means value of the plate agar and turbidity standard curve method follow the first, statistical procedure of two population means of t-Test at α=95% was based the variance analysis. The second, two coefficients of variation (CV %) sample that have \( n_1 \) and \( n_2 \) (\( n>10 \)) size was tested by z value. The CV is

\[
\text{CV} \% = \frac{s \times 100}{m}
\] (3)

where,  
\( s \): standard deviation  
\( m \): mean of data that \( n>10 \)

3. Result and Discussions

3.1. Model discovery

The five datasets merge and were plotted all the fifty bivariate, that have a heteroscedasticity character [18,24]. Verification is confirmation to check that result is correct and consistent. The log transformation data were done for valuable pattern, more interpretable, and helping on inferential statistics for avoid the heteroscedasticity, but the coefficient of determination (r²) was lower on two models (Figure 2).

The transformation choice was helped by knowledge background of the data and it was related to coefficient of regression. That heteroscedasticity character then drives to perform Cook’s D analysis [19] and appears consistent result. The model discovery is turbidity regression of \( S \) cerevisiae in pure water; \( y=bx+a \) and \( x \) is cell number per mL. The one particle of cell in pure water has the turbidity of
1.47 × 10^{-5} NTU. The turbidity value could quantify the cells in pure water and it was verified with colony forming unit (CFU). A Cell quantity using the model of turbidity is analogue with spectrophotometry and both need a standard curve. Therefore the model is a standard curve in turbidimetry.

![Figure 2. Plotting Log Transformation the Predictor Data and Two Trend line Types.](image)

### 3.2. Applying and verification of standard curve

The implemented test result compared two means from equation (1), (2), and CV from equation (3); the viable method by plate agar (PDA) technique for one kind of cell (species) and turbidity method by standard curve technique, value of (log 3.41 × 10^7 ± 3) and (log 2.14 × 10^7 ± 2) were equal significantly (Table 1). The relative standard deviation (RSD) of plate and standard curve of turbidity technique were 13.49 % and 9.03 %. This has mean that the RSD of 9.03% is not requirement the alternate quantification method but RSD of 10-15% is requirement it [25].

| Statistic                  | Plate Method (Log Y) | Turbidity Method (Log Y) |
|----------------------------|----------------------|--------------------------|
| Mean                       | 7.532                | 7.330                    |
| Variance                   | 1.033                | 0.438                    |
| SD                         | 1.016                | 0.661                    |
| Observations               | 19                   | 19                       |
| Pooled Variance            | 0.735                |                          |
| Hypothesized Mean Difference | 0                   |                          |
| df                         | 36                   |                          |
| t Stat                     | 0.725                | 0.236                    |
| P one-tail                 | 0.473                |                          |
| t Critical one-tail        | 1.688                |                          |
| P two-tail                 | 2.028                |                          |
| t Critical two-tail        | 2.758                |                          |
| Coefficient of Variation (%)| 13.494              | 9.029                    |

| z value   | 0.117 |
| P_{z value} | 0.543 |
| D’AD      | 2.758 |
| P_{D AD}  | 0.096 |

The PDA plate showed higher cell number per mL than by standard curve of turbidity technique and calculation method (Figure 3). Standard curve of cell turbidity implementation has lower value of the CV and both method is equal significantly; P=0.543 by z test and P=0.096 by asymptotic (D’AD) test
or hypothesis of \( H_0 : \frac{S_1}{m_1} = \frac{S_k}{m_k} \) is not rejected. The turbidity method use the standard curve is more precise to estimate the \emph{Saccharomyces cerevisiae} cells amount per mL than the conventional method. Those meaning, the turbidity technique that applied the standard curve is alternative for the plate agar technique of viable method.

Figure 3. Box Plot of Cell Number per mL by Turbidity and Plate Methods.

An important preparation for cell counting by turbidimetry is phosphate buffer utilization. This research used ES-4.9PB and 8000g of centrifugation for decreasing aggregate of cell and it was similar procedure with reference [22] which used phosphate saline buffer and 12,000g. A hypothesis, proposition or theory could be test for showing false by an experiment [26]. The experiment for fulfilling the model by hypothesis (\( \alpha \)) is significant equal at 95% confidence. Verification use viable method by plate technique is not standard method of microbiological evaluation in food and nor for index of microbiological diversity in wild nature. That verification method was done by fulfilling that everyone cell can growth and named colony forming unit (CFU) and reference [27] gave the method name microdrop for CFU count. Technically the cell concentration per mL in media is decreased by dilution. Statistical procedure result above showing the one cell is one CFU and it was fulfilled [28]. The cell caused the scattered light is true and the utility of standard curve is true.

3.3. Standard curve implementation for multiple regressions

The implementation of turbidity standard curve was realized to count the cell of \emph{S cerevisiae} that was growing in the potato dextrose broth (PDB) substrate. Researcher implemented the ES-4.9PB dispersant to avoid the aggregate of cells and that application was the preparation of cell counting for the turbidity standard curve. The number of cell in medium (cell.mL\(^{-1}\)), time interval (hours) and the volume of medium were the factor of turbidity medium. Relationship of them result the multiple regressions

\[
Y = -16.609 + 4.079X_1 + 12.257 \times 10^{-6}X_2, \quad R^2=0.877 \quad [29]; \quad \text{where } X_1 \text{ is interval time of cell multiplication and } X_2 \text{ is concentration of cell per mL (Table 2).}
\]

The standard curve coefficient value of \( 1.47 \times 10^5 \) NTU and the multiple regressions of \( 12.257 \times 10^6 \) NTU were equal. The confidence interval (CI) of cell number estimation was used CI method [21] for standard curve and relative estimation error [24]. The CI of coefficient regressions at multiple regressions was based the reference [30]. The result of both values has equal CI respectively; \( (2.96–3.27) \times 10^5 \) and \( (2.72–3.72) \times 10^6 \) cells.mL\(^{-1}\) by those coefficient value. The models were implemented for \emph{S cerevisiae} that the cells were growing in the PDB medium for 13 hours, air flow at 30 °C, and rotated agitation at 75 rpm.
Table 2. The significance and standard error of multiple regression coefficients.

| Factors     | Unstandardized Coefficients | Standardized Coefficients | t   | Sig. |
|-------------|-----------------------------|---------------------------|-----|------|
| (Constant)  | -16.609                     | 7.379                     | -2.251 | .030 |
| 1 time      | 4.079                       | .899                      | .273 | 4.539 | .000 |
| 2 Cells Conc.| 12.257                      | .944                      | .782 | 12.983 | .000 |

a. Dependent Variable: D
Source: Research report [29]

4. Conclusion
The functional relationship of both cell particles and scattered light was significantly linear. However, at the higher cell number in suspended form, the value of NTU unit had a larger error. The regression coefficient of standard curve in turbidimetry is $1.47 \times 10^{-5}$ NTU/cell-$Saccharomyces cerevisiae$ and has relative SE less than 5% at 153 NTU. Follow the CFU verification, the cell quantification using the model on turbidimetry had lower CV and more precise for applying.

Acknowledgment
This research was funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia in year 2018-2019; the higher education superior basic research, letter No.: 3/E/KPT/2018, No.:0045/E3/LI/2018 date January 16th, 2018; in Islamic University of Malang (Unisma) No.: 029/F.05/U/I/LPPM/2018 date January 25th, 2018. The second year, letter No.: 7/E/KPT/2019, No.:113/SP2WLT/DRP/2019, No.:022/SP2H/LT/MONO/L7/2019 date March 26th, 2019 and No: 104/G164/U.LPPM/K/B.07/ IV/2019 date April 1st, 2019.

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