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Research Article

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# METHOD DEVELOPMENT AND VALIDATION OF TOTAL VIABLE COUNT USING SPECIFIED TECHNIQUES AND PERFORMANCE CHARACTERISTICS OF ISO/IEC 17025:2017 IN MICROBIOLOGICAL SAMPLES

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## ABSTRACT

Biofertilizers are microbial based products, and their quality control is very much tricky job due to their biological nature and susceptibility to environmental factors. There are certain parameters that needs to be satisfied for the fitness of product. The principal parameter that showed the presence of microbes in the desired number is Total Viable Count besides other parameters etc. The series of lab studies were carried out at Biofertilizer Testing Laboratory (BTL-FSD), Soil Bacteriology Section, Faisalabad to evaluate the method development and validation characters mentioned in ISO/IEC 17025: 2017 standard for testing laboratories. The method developed keeping in view of (PSQCA standard) PS: 5330/2014 and amended according to the lab conditions and validated. Different techniques mentioned in clauses 7.2.2 and 7.2.2.3 were carried out in the lab environment. The bias expressed as LOG 10 were within the range of -0.024 to 0.034 and relative standard deviation (RSD) was <2%. The RSD of repeatability was 0.607% and reproducibility was 0.656 and 0.744%. The significance determined as t-stat was 0.280 well below the t-critical one and two tail i.e., 1.86 and 2.31, respectively and accuracy of method using robust mean was 99.8%. The measurement of uncertainty (MoU) was ±0.05 at 95% confidence interval and expanded uncertainty of the method was 0.1145. The LOD and LOQ of the method was evaluated as 0.090897 and 0.275445 expressed as LOG 10. On the basis of techniques and characters under assessment, it was concluded that method of total viable count satisfied the requirements of ISO/IEC 17025:2017 standard and validated.

**Keywords:** Performance characteristics, validation techniques, precision, accuracy, LOD, LOQ, linearity, robustness, total viable count, microbiological method.

## INTRODUCTION

Soil and agriculture related microbiology needs high need of expertise and advancement in analytical ability for registration and quality control purposes. The microbiological methods are vulnerable to high risk of variation due to their biological nature of samples and required optimum conditions for their analysis. The microbiological method development is a difficult task because it needs to address the variations that can contribute to misleading values. The method advancements / innovation leads to substitute method techniques that can be addressed by the concerned analysts. The automatic scientific instruments should be used with more precise care following the required protocol and consequently the results. The development of method should be carried out keeping in view the potential contaminants such as air, water, sterilizing instrument's efficacy, lab environment and staff. The microbiological method should have flexibility to cope with the potential changes / variations opted by the analysts during their analytical work. The contamination especially of microbial nature during microbial assessments is very much critical because of microbial competition for the nutrient sources especially N and C and affected the resultant output. The avoidance of microbial contaminants is the prerequisite for the positive and accurate result. The microbiological method should be "SMART means specific, measurable, achievable, realistic and time bound" (AOAC, 2006; Eurachem, 2013; Sandle, 2015). The method used in microbiological analysis should be accurate, precise, flexible and sample preparation methodologies are trustworthy and reliable (AOAC, 2006; Kretzer *et al.,* 2008; Aboul-Enein and Sibel, 2012; Tijare *et al.,* 2016).

The microbiological estimation or detection is highly relied on the sample quality and their preparation for analysis. These dependencies are numerous such as samples and their matrices are heterogenous in nature, incompatibility of carriers, the potential target microbes are of single type or multiple

origins or their competition, mixed or pooled samples, and the method that has been under consideration might have required very minute amount for analysis. The method development is crucial step for proper outcome of results and tackle any intervention during the production of viable and accurate results. These method developments, verification and validation are carried out to produce the valid results. According to guideline (ILAC-G9, 2005), the accurate and valid analyses or measurements depends upon the points that must be kept in consideration viz. the accuracy of developed, verified / validated method and usage of apposite calibrated instruments, the usage of reference materials or certified reference materials meeting the ISO-17034 requirements (ISO 17034, 2016), the competence of lab staff or analyst, assuring the equivalence of analyses means traceability, third party evaluation like inter-lab comparison and proficiency testing meeting the ISO-17043 standard (ISO 17043, 2016), uncertainty measurements taken into account, the employing precise quality control assurance policies / undertakings / plans / procedures and achieved accreditation from a accreditation body (ISO/IEC 17025, 2017).

The microbiological testing or analyses is known worldwide and comprised of isolation, multiplication, enumeration, identification of microbes or their primary or secondary metabolites and detected vs not-detected (NELAC, 2007; Sandle, 2014; CDER, 2015). There are three types of microbiological methods viz. i) Qualitative testing for presence or absence of methods e.g. presence or absence of *E. coli* or *coliforms* and others ii) Quantitative testing for direct enumeration of target microbe e.g. total viable count or colony forming units (CFU) or indirect enumeration i.e., most probable number (MPN), color development / absorbance in the given sample (NordVal, 2009, Eurachem, 2013), iii) microbial identification test either biochemically or by using DNA sequencer and various other mechanisms such as cell wall pattern, carbon utilization tendency, growth hormone production potential and biofilm production etc. (Sutton, 2005; Sandle, 2015; Duygu and Udoh, 2017). The microbiological methods performed qualitatively, the method should be verified by suitability, specificity, deviations either positive or negative, precision parts as repeatability and reproductivity etc. while microbiological methods performed quantitatively where the results are reproduced in numeric number, the method should be verified by specificity, sensitivity, deviations either positive or negative, repeatability, reproducibility, limit of detection / quantification, uncertainty measurement etc. and the techniques of method validation and performance characteristics as laid out in ISO/IEC 17025:2017 and ISO 7218:2007 (ISO 13528, 2005; ISO 7218, 2007; ISO/IEC 17025, 2017; Duygu and Udoh, 2017).

The developed method can only be qualified as candidate method for producing the valid results if the method is validated. Hence, validation is the series of processes or characteristics performed in the lab by following the techniques or performance characteristics of method that fulfills the intended purpose. Therefore, the method validation is a processed information when applied to an analytical or microbiological method to meet the intended purpose for required standard's demand. The ultimate objective of method-validation is that method will perform outstandingly for the entire finding of experimental results.

The method validation of microbiological methods should be carried out in such a way that sample under analysis should not have any intrinsic element that hampers the growth of microbial cells and upon culturing the required amount can be obtained (NELAC, 2007; Eurachem, 2012, 2014; Sandle, 2015; Duygu and Udoh, 2017; Bramwell *et al.,* 2022). The method validation is of two types i.e., primary validation and secondary validation. The microbiological analysis, either qualitative or quantitative, the methods are divided into three types i.e., standard method, rapid method and non-standard method. The standard method is the one that employs only verification and lab should ensure its competence regarding the performance characteristics. The rapid method is sometimes called as kit method or on-site method that is used under the prevailing environmental conditions and generally employed only verification of method and rarely applied detailed assessment verified by lab determined results compared with kit method. The non-standard method are unpublished, lab developed / designed methods or the methods that have been amended according to the lab environment needs primary validation and annual confirmation by verification. Before performing the primary validation, the lab should develop quality assurance plan or validation protocol that mentioned the suitability by evaluating with other standard methods or inter-lab studies and must meet the desired need of the project. The lab developed method or modified standard method should fulfills the minimum requirement of ISO standard (ISO/IEC 17025:2017) for different performance characteristics "accuracy, precision, repeatability, intermediate precision, reproducibility, sensitivity / specificity, linearity, limit of detection / quantification etc". The validation of microbiological methods is critical for producing high quality valid results (AS 5013.14.1, 2010; Sereia *et al.,* 2017; Arkaban *et al.,* 2021).

Biofertilizers / microbial inoculants are comprised of beneficial living organisms that colonizes the plant root surface or rhizosphere and exert their effect by promoting the growth of plants. Biofertilizers contains rhizobacteria termed as PGPR "plant growth promoting rhizobacteria", and can promote growth of plants by direct vs indirect mechanisms (Kumar *et al.,* 2020; Liu *et al.,* 2022; Qureshi *et al.,* 2023). These biological formulations improve the plant growth and overall productivity by nutrient fixation / solubilization, improving nutrient uptake, enhancing

nutrient use efficiency and producing plant hormones. The provision of quality biofertilizers to the end users is the prime factor results in their positive impact and acceptance / rejection by the farmers. The quality relates with the presence of target microbes in active form in the said product. The primary parameters that illustrate the quality biofertilizers are total viable count at time of manufacturing or expiry, contamination level, pH, moisture and efficiency character of isolate etc. The total viable count of biofertilizers is the principal and crucial factor that ensures the biofertilizer's quality. The standard operating procedure (SOP) of total viable count needs to be confirmed or validated and assuring that method is fit for intended purpose. The following validation techniques in ISO/ IEC 17025:2017 under clause 7.2.2 were mentioned and discussed.

As far as the technique of "Calibration or evaluation of bias and precision using reference standards or reference materials" is concerned, the details are given below,

The calibration of equipment's by third party reference laboratory having accreditation of ISO standard 17025, after comparison of reference lab standard to the testing lab, a value is ascribed on the calibration certificate for further estimations by the testing lab. Calibration leads to accuracy of instruments and ultimately accurate and precise measurements (SIO. There are many types of calibration i.e., temperature, pressure, electrical, flow etc. The principal impact of calibration is that to maintain accuracy, precision, instrument variations and standardizations evaluated by the measurement of uncertainty (Stephen, 2003; ISO Guide 99, 2007; Panhwar *et al.,* 2020).

Bias can be described as the "difference between the reference value and measurement average by the testing lab in quantifiable form". The testing lab's mean difference to the reference lab's mean over particular time span for the same item can be reduced by calibration of apparatus and equipment's or by in-house calibration to the reference standard at regular intervals (Theodorsson *et al.,* 2014).

Precision is termed as "mean of set of measurements expressed as Repeatability, Intermediate precision and Reproducibility". Precision usually deemed at different levels i.e., within-lab repeatability and reproducibility, between-lab repeatability and reproducibility. The primary validation usually covered the first two levels over the concentration range of the said sample (US EPA, 2009; Chesher, 2008; Barnawal *et al.,* 2016).

Repeatability is defined as "the nearness of test results acquired from the same method but conditions are similar as test item, lab, analyst and equipment within short intervals of time". Repeatability is also designated as intra-assay precision.

Intermediate precision (within lab reproducibility) is dissimilar from repeatability, the precision obtained from a lab over time span and have more variations than repeatability. Intermediate precision was evaluated by performing the analysis in the same laboratory on two separate days with different analysts. Reproducibility can be defined as "the nearness of test results of same sample carried out under variable conditions". The reproducibility means obtaining results from different labs. The single lab validation does not need reproducibility rather it is advantageous if method has to be used in more than one lab or the method has to be standardized.

As far as the description regarding "b) Systematic assessment of the factors influencing the result", the assessment of factors that influence the results should be kept in consideration while producing the results. The random or systematic errors that affect the analytical results should be evaluated. The combination of random or systematic errors produces the total errors. The estimation of random errors is carried out as precision and estimation of systematic errors as trueness and expressed as standard deviation. After the calculation of uncertainty budget, the standard deviation expressed as expanded uncertainty of the method. The systematic assessment of factors that contributed in terms of uncertainty while making the uncertainty budget are repeatability, reproducibility, incubator, analytical balance, pH meter, oven, autoclave, micropipette, environment temperature and moisture etc.

The description regarding "c) Testing method robustness through variation of controlled parameters, such as incubator temperature, volume dispensed", the method robustness is the capability of method to produce the almost similar results keeping in view the minor deviations in the method parameters. The robustness also reflects the method adaptability in more than one lab. If minor changes in the method have to be carried out according to prevailing lab environment, the method should be robust enough to produce the comparable results.

The description regarding "d) Comparison of results achieved with other validated methods", the comparison of results is aimed to find the difference between the more than one methods / procedure. If the results produced by one method of similar type is compared with other one, the difference is attributed by sample handling, sampling technique, changes in procedure main part, environmental factors may contribute to larger variations. The comparisons of method can be carried out by participating in Inter-Lab Comparison (ILC), Intra-Lab Comparison, Proficiency Testing (PT) etc. (Hanneman, 2008; Pharmeuropa, 2015). As far as the technique "e) Inter Laboratory Comparisons", the Inter Lab Comparisons evaluate the competence of participating labs. This practice ensures the overall performance of lab and provides an opportunity for the analytical ability of lab staff, for comparison of methods followed in the participating labs and improve the confidence / competence of lab staff. For the technique of "f) Evaluation of measurement uncertainty of the results", the

measurement of uncertainty is a quantitative value that reflects the reliable results and measurement standard. The measurement uncertainty is the statistical dispersion and showed the interval between tested and true value (Veen and Cox, 2021). The measurement uncertainty (MU) is defined by ISO 15189 (item 3.17) as "a parameter associated with the result of a measurement that characterizes the dispersion of values" (ISO 15189, 2012; NATA, 2012; Schneider *et al.,* 2017; Chaudhary *et al.,* 2021; IUPAC, 2023).

According to the prevailing standard for testing laboratories i.e., ISO/IEC 17025:2017, the standard method if amended needs to be validated to confirm that it justifies the required criteria and serves for the intended purpose. The preamble of the present study was to validate the method of total viable count of biofertilizers by using the specified techniques and performance characteristics.

## MATERIALS AND METHODS

The lab used standard dilution plate technique for determining the total viable count in biofertilizers and followed the method given by Pakistan Standards Quality Control Authority (PSQCA) for biofertilizers (PS: 5330 / 2014). The most extensively used method for viable cell count consists of diluting biofertilizer sample with sterile saline solution until the bacterial cells are diluted sufficiently to count accurately. The method validation was carried out using techniques of method validation as laid out in ISO/IEC standard 17025:2017 clause 7.2.2 and performance characteristics in clause 7.2.2.3 includes range, accuracy, measurement uncertainty of results, limit of detection (LOD), limit of quantification (LOQ), method selectivity / specificity, precision (Repeatability, intermediate precision or reproducibility), method robustness and bias etc.

**Bias:** Bias can be assessed by the difference of mean of measured values to mean of reference values. Bias is calculated by as Bias = $\bar{x}$ - μ, where $\bar{x}$ = the average of measurements by the testing lab and $\mu$ = the average of measurements by the Reference standard. When Bias was expressed in percentage then calculated by Bias (%) = $\bar{x}$ - μ / μ x 100 (Theodorsson *et al.,* 2014).

**Precision:** Precision is usually expressed as repeated measurements displaying similar results under un-changed conditions. Precision portrays the nearness of measured values from actual (true) value and is usually expressed as the variance / standard deviation, or coefficient of variation (CV) or relative standard deviation (RSD), where: CV or RSD (%) = (standard deviation of measurements / mean) x 100

Precision was measure of repeatability, intermediate precision / reproducibility and determined as standard deviation and relative standard deviation (ISO 21748, 2010; Barnawal *et al.,* 2016; Chaudhary *et al.,* 2021, IUPAC, 2023).

**Accuracy:** The accuracy of a microbiological method is the "nearness to the test results to the true value" (CDER, 2015). The accuracy of method is combination of random errors, while bias related to the systematic errors correlated with the method (ISO 5725, 1994a). The ISO standard 5725 used the terms 'trueness' and 'precision' to elaborate the method accuracy. The 'Trueness' denotes the nearness of arithmetic mean of test results and reference value while 'Precision' mention the nearness between the test results (ISO 5725, 2023). The spiking of samples usually carried out to produce the accurate results. The mean value of repeated measurements of spiked materials with standard deviation compared with illustrated value as reference. The spiking of microbiological samples can be carried out or by comparing one method with other method. The spiking of samples expressed as percent recovery or relative recovery means measured value divided by assigned value x 100 (ISO, 2000). Accuracy can also be measured in terms of error percentage (Desta and Amare, 2017; Sinshaw *et al.,* 2019) i.e., Accuracy = 100– error.

Where Error (%) = Measured value – Reference value / Reference value x 100

The degree of accuracy or comparison of accuracy of method for some samples can be assessed by Significance t-test. Accuracy can be measured as Accuracy % (Sandel, 2015)

Accuracy % = (Number of Correct Results in Agreement/Total Number of Results) x 100

**Specificity:** The specificity of a quantitative microbiological method is its ability to detect a panel of microorganisms suitable to demonstrate that the method is fit for its intended purpose. This is demonstrated using the organism's appropriateness for the purpose of the alternate method. Specificity is the capability of the method to resolve or measure a range of microorganisms in the presence of other compounds or microorganisms. Specificity of microbiological testing method is evaluated by analysing the blank samples (carrier based and liquid based). The blank sample is that sample that has no analyte. The BTL-FSD has prepared the blank sample of biofertilizer of both types and sterilized in a calibrated autoclave. The sterilized samples have been treated as biofertilizer sample with the same method and at the same time autoclaved distilled water was also used. No growth of microbe was detected (INAB, 2012; Eurachem, 2014; Duygu and Udoh, 2017).

**Uncertainty Measurement:** Uncertainty is a quantitively derived value and is deviation from the reference value and expressed in the form of range appeared ± after sthe reported value. The measurement of uncertainty is an interval symbolizes the dispersion of values related to results and reflected the range of errors or standard deviation. The Health Protection Agency (2005) evaluated the sources of uncertainty from microbiological methods were competence and variations between analysts, sample status and homogenization, dilutions, media, inoculation method and interpretation of results. The uncertainties in the test results might be contributed by numerous factors such as analysts (Repeatability, reproducibility),

method variations, apparatus, chemicals, environmental factors i.e., temperature and humidity, and equipment's etc.

Various tactics were used for the calculation of uncertainties that should be kept in consideration while preparing the uncertainty budget, including precision (repeatability and reproducibility) of the method, bias with reference material uncertainty, uncertainty of each apparatus and equipment's from the calibration certificates or any factor that may hamper the validation output etc (JCGM, 2008; Magnusson *et al.,* 2017). The lab apparatus and equipment's uncertainties were derived from their calibration certificate. The uncertainty of each factor was cumulated to form combined uncertainty and ultimately form the uncertainty budget. As per standard (ISO/IEC 17025:2017), the testing labs should demonstrate uncertainties with designated confidence level i.e., Expanded Uncertainty and lab used confidence level of 68% to calculate uncertainty (ISO 21748, 2010; Eurachem, 2014; Farrance *et al.,* 2018; Nazir *et al.,* 2020; Aslam *et al.,* 2021; Veen and Cox, 2021; Ullah *et al.,* 2022; IUPAC, 2023).

**Linearity:** According to ICH guideline (ICHQ2(R1)) the linearity of an analytical method can be explained as *"results that are directly proportional to the concentration of the analyte in the sample"* (ICH guideline, 2005). Linearity is often measured within a given range. Linearity is a mathematical relationship between two variables which are directly proportional to each other. The BTL-FSD used 1.0 g sample of biofertilizer for the preparation of serial dilutions as per SOP, so linearity of the total viable count method was established by using the biofertilizer sample weights in grams i.e. 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2. The Regression equation or trendline equation was applied for linearity calculation and regression coefficient or coefficient of determination ($R^2$) was measured.

**Limit of Detection / Quantification:** The limit of detection (LOD) and limit of quantification (LOQ) are important performance characteristics in method validation. The lowest content of analyte that can be detected is the limit of detection and not quantified while the lowest amount that can measured or quantified is the limit of quantification (McDowall, 2005; González *et al.,* 2010; González *et al.,* 2018). The lab appraised quality of its analytical methods in terms of suitability for its intended purpose. The LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. The lab evaluated LOD & LOQ involving the Regression Chart by the following equations,

LOD = 3.3 x (SD of Intercept/Slope)

LOQ = 10 x (SD of Intercept/Slope)

**Robustness:** The lab followed Dilution Plate Technique for the determination of total viable count (TVC) of biofertilizer sample. The method used plating method was spread plate. The other famous method was pour plate was used to judge the robustness of the method. In dilution plate technique, serial dilutions were prepared in sterilized distilled water and the amount of dilution dispensed on the agar plate were varied to assess the robustness of method. The method adopted by the BTL-FSD, incubated the petri plates at 28 ± 2ºC and 30 ± 2ºC for 48 hours to check the robustness of the method. The total viable count was calculated the number of cells (CFU) per mL or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar. The robustness of the testing method was carried out with two plating methods i.e. spread and pour plate and two levels of volume dispensed / amount plated i.e. 0.1 and 0.2 mL at two temperature levels i.e. 28 ± 2ºC and 30 ± 2ºC for 48 hours.

## RESULT AND DISCUSSION

Different techniques and performance characteristics were evaluated for the validation of method regarding total viable count in biofertilizer samples such as bias, precision (bias, precision (repeatability and intermediate precision/ reproducibility), accuracy, specificity, uncertainty measurement, linearity, limit of detection/quantification, inter lab analysis, proficiency testing etc.

The biasing of the microbiological method was determined from the mean of measured value and Robust mean from the reproducibility mean using standard deviation. The biasing of method was determined using the Inter Lab Comparison (ILC) reported result value and overall mean value and results are reported with Z score and proved as passed. At the same time lab value of Proficiency Testing (PT) of water sample and assigned value of for aerobic plate count and heterotrophic plate count and results are reported with Z score and proved as passed. The biasing of method of total viable count of biofertilizer sample in Table 1 was determined using the reproducibility mean expressed in LOG 10 i.e., 8.266 and bias values were within the range -0.024 to 0.034. The Z Score of the both the analyst was calculated and it was within the satisfactory range i.e., ±2.0. Moreover, the RSD was also determined for both analysts and mean values and it was <2%. Results obtained clearly suggested that the method produced satisfactory results and proved to be passed. The biasing of PT (water samples) and ILC (biofertilizer samples) in Table 7 was carried out and illustrated the difference of lab results and PT/ILC assigned and mean values in the form of Z score and results were found within the range of required Z score i.e., ±2.0 (Shabir, 2004; Ullah *et al.,* 2022; IUPAC, 2023).

The lab checked the precision in terms of Repeatability, Intermediate Precision and/or Reproducibility. For repeatability, the sample was prepared / processed / replicated and same analyst recorded readings while for the Intermediate Precision /reproducibility, the same sample was prepared /

processed / replicated and two analysts-1 and analyst-2 recorded the readings. The repeatability of total viable count (TVC) from biofertilizer sample was carried out under similar set of conditions i.e., by same analyst using same apparatus, lab and within short span of time. The same analyst again repeated the set under identical set of conditions. The value of total viable count / gram of sample was converted to Log 10 and standard deviation, relative standard deviation (RSD) and percent or relative standard deviation was evaluated (RSD%). The repeatability results presented in Table 2 demonstrated that relative or percent standard deviation was 0.607% and well below the acceptance level i.e., 2%. This clearly indicated that developed method was produced reproducible results and hence termed as passed (González *et al.,* 2010; Eka *et al.,* 2012; Ullah *et al.,* 2022).

The determination of intermediate precision / reproducibility was performed by two analysts at different time span demonstrated in Table 3 for analyst-1 and analyst-2, the RSD% was 0.656 and 0.744%, respectively. The RSD was pooled for both analysts and that was 0.7% and well below the acceptance level i.e., 2%. The reproducibility data was analysed statistically using t-Test by MS Excel data analysis and found that t-stat was 0.280 well below the t-critical one and two tail i.e., 1.86 and 2.31, respectively. Thus, the results produced were non-significant to each other and yielded reproducible results and considered as validated method (Ullah *et al.,* 2017; IUPAC, 2023).

The accuracy of method of total viable count was determined by dividing the measured value to the reference value after spiking of sample as Robust mean (X-estimate) achieved after incorporating the standard deviation. The lab also checked the accuracy of PT of water sample for Aerobic Plate Count as lab value divided the assigned value x 100.

Mean value = 7.827
X Estimated Mean (Robust Mean) =
7.841
Accuracy= 99.8%

The measurement of uncertainty (MoU) in Table 6 was determined using the standard deviation of repeatability, reproducibility for type-A uncertainty and uncertainty of each instrument used in the method and environmental factors like temperature and humidity for type-B uncertainty. Firstly, the combined uncertainty was cumulated by combining all the mentioned above uncertainties and then expanded uncertainty by multiplying the combined uncertainty with confidence interval. The MoU of the method under validation was ±0.05 at 95% confidence interval. Above mentioned measurement of uncertainties is summarized in Uncertainty Budget to evaluate the combined and Expanded Uncertainty of the method i.e., 0.1145 and given in Table 6. The evaluation of the MoU which remains less than 5% indicated that the method adopted for determining the TVC in Biofertilizer samples is FIT for the intended purpose (González and Herrador, 2007; Magnusson *et al.,* 2017; Farrance *et al.,* 2018; Sunilkumar *et al.,* 2020).

The linearity was evaluated by taking sample weights and viable count value expressed as LOG 10 in Table 4. For linearity calculation, Regression and trend-line equation was applied and regression coefficient or coefficient of determination ($R^2$) measured i.e., 0.9996. From the values of linearity was established, the standard error (SE) was evaluated using MS Excel data analysis, Regression and standard error value obtained that was used for standard deviation as SE of Intercept multiplied by the square root of number of values (n). The LOD and LOQ in this assay of method validation was found as 0.090897 and 0.275445 expressed as LOG 10 of total viable count (González *et al.,* 2010; Renger *et al.,* 2011; Chaudhary *et al.,* 2021). The LOD and LOQ was calculated by incorporating slope of regression equation (Sedlak and Paprštein *et al.,* 2011; Magnusson *et al.,* 2017; Farrance *et al.,* 2018; Gudžinskaitė *et al.,* 2020; Chaudhary *et al.,* 2021) using formula mentioned below,

LOD = 3.3 x (SD of Intercept/Slope)
LOQ = 10 x (SD of Intercept/Slope)

The Table 5 regarding the robustness of the testing method demonstrated that both plating methods and variable level of amount dispensed and at different incubation temperatures illustrated almost similar relative standard deviation of log 10 values of TVC against each parameter i.e., plating method, incubation temperature, and amount dispensed on petri plates., respectively was < 2%. The validation assay for robustness clearly indicated the tendency of method for flexible results such as RSD (method) for spread and pour plate method i.e., 0.004 and 0.008 at 28±2 ºC, respectively while similar results were obtained at 30±2 ºC for both methods i.e., 0.011 and 0.009, respectively. The RSD for temperature was found to be 0.005 and 0.009 at both temperatures. i.e., 28±2 and 30±2 ºC and well below the acceptance level i.e., < 2% (Health Canada, 1994; Green, 1996; Shabir, 2004; Eurachem, 2014; Tijare et al., 2016; Duygu and Udoh, 2017; FDA, 2019; Aslam *et al.,* 2021).

**Table 1.** Evaluation of Bias by using SD and reproducibility mean

| Repeats | Analyst-1 | Analyst-2 | Reproducibility Mean | Biasing | Z Score |
|---|---|---|---|---|---|
| 1. | 8.255 | 8.230 | 8.243 | -0.024 | -1.022 |
| 2. | 8.230 | 8.279 | 8.255 | -0.012 | -0.502 |
| 3. | 8.255 | 8.301 | 8.278 | 0.012 | 0.515 |
| 4. | 8.279 | 8.322 | 8.301 | 0.034 | 1.489 |
| 5. | 8.255 | 8.255 | 8.255 | -0.011 | -0.481 |
| Mean: | 8.255 | 8.277 | 8.266 | | |
| SD: | 0.0173 | 0.0364 | 0.0231 | | |
| RSD: | 0.210 | 0.440 | 0.280 | | |
| Max SD: | 0.0364 | | | | |
| X Estimate: | 8.277 | | | | |

**Table 2. Repeatability of total viable count of biofertilizer sample.**

| Number of Repeats | Total viable count / gram |
|---|---|
| | Log 10 |
| 1. | 7.839 |
| 2. | 7.875 |
| 3. | 7.924 |
| 4. | 7.954 |
| 5. | 7.857 |
| Mean: | 7.890 |
| SD: | 0.048 |
| RSD: | 0.006 |
| RSD (%): | 0.607 |
| Acceptance Criteria: | %RSD ≤ 2% |

**Table 3. Intermediate Precision / Reproducibility of total viable count of biofertilizer sample.**

| Number of Repeats | Total viable count / gram | |
|---|---|---|
| | Analysts-1 | Analysts-2 |
| | LOG 10 | LOG 10 |
| 1. | 7.869 | 7.792 |
| 2. | 7.924 | 7.903 |
| 3. | 7.799 | 7.881 |
| 4. | 7.892 | 7.944 |
| 5. | 7.924 | 7.839 |
| Mean: | 7.882 | 7.872 |
| SD: | 0.052 | 0.059 |
| RSD: | 0.0066 | 0.0074 |
| RSD%: | 0.656 | 0.744 |
| RSD Pooled: | 0.007% | |
| RSD Pooled (%): | 0.70% | |
| Acceptance Criteria: | %RSD ≤ 2% | |
| t-Test (stat): | 0.280 | |
| T Critical one tail: | 1.86 | |
| T Critical two tail: | 2.31 | |

**Table 4. Validation assay sheet for linearity and estimation of LOD and LOQ.**

| Weight of sample | TVC in LOG10 |
|---|---|
| 0.2 | 5.35 |
| 0.4 | 6.05 |
| 0.6 | 6.72 |
| 0.8 | 7.38 |
| 1.0 | 7.95 |
| 1.2 | 8.60 |
| Mean: | 7.01 |
| SD: | 1.2101 |
| n: | 6 |
| Slope: | 3.2327 |
| Intercept: | 4.7461 |
| Correlation Coefficient (r): | 0.9996 |
| Linearity range: | 0.2-1.2 |

| | |
|---|---|
| SE of Intercept: | 0.036351755 |
| SD of Intercept: | 0.089043252 |
| LOD (LOG 10): | 0.090897 |
| LOQ (LOG 10): | 0.275445453 |

**Table 5. Validation assay to find the Robustness of the TVC method**

| Incubation Temp | Plating Methods | Amount Plated (mL) | LOG 10 | RSD (Method) | RSD (Temp) |
|---|---|---|---|---|---|
| 28±2 | Spread Plate | 0.1 | 7.92 | 0.004 | 0.005 |
| | Spread Plate | 0.2 | 8.18 | | |
| | Pour Plate | 0.1 | 7.94 | 0.008 | |
| | Pour Plate | 0.2 | 8.16 | | |
| 30±2 | Spread Plate | 0.1 | 8.03 | 0.011 | 0.009 |
| | Spread Plate | 0.2 | 8.20 | | |
| | Pour Plate | 0.1 | 8.00 | 0.009 | |
| | Pour Plate | 0.2 | 8.20 | | |

**Table 6. Measurement of Uncertainty (MoU) evaluation or total viable count method.**

| S/N | Sources of Uncertainty | Uncertainty | Type A/B | K Factor (Where Applicable) | Uncertainty Contribution | Average or Value | Relative Uncertainty | Combining Uncertainty |
|---|---|---|---|---|---|---|---|---|
| 1 | Repeatability | 0.0173 | A | 1 | 0.0173 | 8.255 | 0.0020957 | 4.39196E-06 |
| 2 | Reproducibility | 0.0364 | A | 1 | 0.0364 | 8.277 | 0.004397729 | 1.934E-05 |
| 3 | Incubator | 1 | B | 2 | 0.510204082 | 28.0 | 0.018221574 | 0.000332026 |
| 4 | Micropipette | 0.1 | B | 2 | 0.051020408 | 1.000 | 0.051020408 | 0.002603082 |
| 5 | Analytical Balance | 0.0001 | B | 2 | 5.10204E-05 | 10.0 | 5.10204E-06 | 2.60308E-11 |
| 6 | pH Meter | 0.01 | B | 2 | 0.005102041 | 6.99 | 0.000729906 | 5.32762E-07 |
| 7 | Oven | 1 | B | 2 | 0.510204082 | 105.0 | 0.004859086 | 2.36107E-05 |
| 8 | Autoclave | 1 | B | 2 | 0.510204082 | 121.0 | 0.004216563 | 1.77794E-05 |
| 9 | Environment Temperature | 0.6 | B | 2 | 0.306122449 | 25.0 | 0.012244898 | 0.000149938 |
| 10 | Environment Moisture | 1 | B | 2 | 0.510204082 | 45.0 | 0.011337868 | 0.000128547 |
| | **Combined Uncertainty (Uc)** | 0.057264714 | @ | **68 % CL** | | | | |
| | **CL (K)** | 2 | = | **95% CL** | | | | |
| | **Expanded Uncertainty (Ue)** | 0.114529428 | @ | **95% CL** | | | | |

**Table 7.** Biasing in the PT and ILC samples for estimation of Cell Count

| TVC in PT /ILC Samples | Assigned / Mean Value (LOG 10) | Lab Value (LOG 10) | Z score (LOG 10) | Remarks |
|---|---|---|---|---|
| Enumeration of Aerobic Plate Count (NPSL, Islamabad); PT Round MW-09; Issue date: January, 2021. | 3.27 | 3.56 | 0.82 | Satisfactory |
| Enumeration of Heterotrophic Plate Count 35±1ºC (NPSL, Islamabad); PT Round MW-10; Issue date: January, 2021. | 3.00 | 3.40 | 1.12 | Satisfactory |
| Enumeration of Total Plate Count (NPSL, Islamabad); PT Round MW-11; Issue date: January, 2022. | 2.81 | 3.34 | 1.53 | Satisfactory |
| Enumeration of Heterotrophic Plate Count 35±1ºC (NPSL, Islamabad); PT Round MW-12; Issue date: January, 2022. | 3.00 | 3.06 | 1.41 | Satisfactory |
| Heterotrophic Colony Count 35±1ºC (NPSL, Islamabad); PT Round MW-14; Issue date: October, 2023. | 5.534 | 5.531 | -0.75 | Satisfactory |
| Heterotrophic Colony Count 23.5±1.5ºC (NPSL, Islamabad); PT Round MW-1; Issue date: October, 2023. | 5.4314 | 5.4310 | 0.53 | Satisfactory |
| TVC of Biofertilizer; ILC organized by Provincial Fertilizer Testing Lab, Lahore; Issue date: December, 2019-20 | 8.329 | 8.114 | -0.81 | Satisfactory |
| TVC of Biofertilizer; ILC organized by Provincial Fertilizer Testing Lab, Lahore; Issue date: August, 2020-21. | 8.804 | 9.875 | 1.10 | Satisfactory |
| TVC of Biofertilizer; ILC organized by Provincial Fertilizer Testing Lab, Lahore; Issue date: August, 2022-23 | 7.926 | 8.301 | 0.69 | Satisfactory |
| TVC of Biofertilizer; ILC organized by Provincial Fertilizer Testing Lab, Lahore; Issue date: June, 2023-24 | 8.366 | 9.544 | 0.82 | Satisfactory |

## CONCLUSION

Othe basis of validation techniques adopted and performance characteristics carried out in the lab, it was concluded that total viable count testing method satisfied the requirements of ISO/IEC 17025:2017 standard. The opted method is simple, easy to perform and produce accurate, precise and valid results.

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