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RESEARCH ARTICLE

VALIDATION OF TEST METHODS FOR COMMERCIAL RELEASE OF PROBIOTIC *BACILLUS CLAUSII* SPORES AND SPORE SUSPENSIONS

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ABSTRACT

Researchers and manufacturers are focusing on the development of probiotic formulations because of consumers' awareness. By considering this, regulators are framing guidelines for ensuring the safety of live probiotics. *Bacillus clausii* with various formulations has a major share in the current market. We are Elmed Life Sciences, a leading probiotic manufacturer, manufacturing *Bacillus clausii* in different formulations. To ensure the quality and safety of our products, we adopted and modified test parameters like pH, Gram staining, spore staining, motility test, total viable count, heavy metals, arsenic, specific pathogens, and total yeast and mold count, validated and verified as per ICH Q2R2 guidelines for its intended purpose of use. All the test parameters met predefined validation criteria with respect to the coefficient of variation for intra-assay, inter-assay precision, limit of detection, recovery for accuracy, limit of quantification, robustness, coefficient of determination for linearity, range of the assay, and method specwificity for the specificity test, and reported the lower and upper limits of detection and quantification. We conclude that these parameters were suitable for testing and commercial release of the *Bacillus clausii* spores and its formulations.

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INTRODUCTION

A live beneficial microbiota or probiotic microorganism plays an important role in balancing the animal gut flora, a healthy digestive system can modulate all other functions of the animal system (Sarita *et al*., 2025). Because of consumer awareness about the beneficial effects of probiotics, the global market in the area of probiotics is growing at a compound annual growth rate of 14% from 2024 to 2030 (Probiotic market, 2024). Elmed life sciences (ELS), for ensuring the safety and efficiency of products adopted testing methods from the various pharmacopeia and established research data. The suitability of those methods was proven in this research work. Validation of analytical methods in the pharmaceutical industry is to demonstrate the selected methods are specific and produce accurate results to confirm the quality of the product manufactured and released for commercial applications (ICH Q2R2, 2023). To ensure the safety and efficiency of the probiotics many regulatory agencies framed guidelines for selection, screening, characterization, manufacturing, formulations, and quality testing. Reputed regulators like United States Pharmacopeia (USP), British Pharmacopeia (BP), European Pharmacopeia (EP), Indian Pharmacopeia (IP), etc. established common guidelines for validation of analytical methods for uniformity. Authors referred various guidelines like validation of analytical procedures (ICH Q2R2, 2023), Pharmaceutical quality system (ICH Q10, 2008), analytical procedures and methods validation for drugs and biologics, method verification and validation (FDA, 2015), Bioanalytical method validations guidance for industry (FDA, 2018), Verification of compendial procedures (USP<1226>), Validation of compendial procedures (USP<1225>) and Validation of alternative microbiological methods (USP<1223>). Based on these guidelines ELS prepared standard operating procedure and validation protocols, executed the protocols using commercial test samples like CSS09624, CSS09724, CSS09824, BCS00125, BCS00225 and BCS00325; *Bacillus clausii* lyophilized powder/Active pharma ingredient batch numbers like BCD01524, BCD01624, BCB00125 and BCB00225 which were isolated and characterized as per the modified procedure of Bhima *et al*. (2019) and Srinivas *et al*. (2017) and report has been prepared, approved and archived at quality assurance department. Specificity of the method confirms that the analyte of interest in the formulation can be identified or quantified without the interference of the excipients expected to be present in the formulation. It is also demonstrated by discrimination of the analyte in the presence of impurities and/or excipients. The discrimination of a method may be confirmed by obtaining positive results from samples containing the analyte, coupled with negative results from

samples that do not contain the analyte. The specificity of the test method will also be evaluated by comparing the specificity results of the placebo with the standard spiked in the test sample (Qureshi *et al*., 2024; ICH Q2R2, 2023; Duygu *et al*., 2017; Zwietering *et al*., 2016). The precision of an analytical procedure expresses the closeness of agreement between various concentrations of the sample, analysts, laboratories, analysis time, and within the same strength of the sample analyzed in multiple replicates (Bollmann, 2016; Bertrand *et al.*, 2005). The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (Qureshi *et al*., 2024; Sahitya *et al*., 2020; Duygu *et al.*, 2017; Hospodsky *et al*., 2010). The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of the analyte of interest in the sample over the range of the method (Qureshi *et al*., 2024; Sahitya *et al*., 2020; Duygu *et al*., 2017). The quantitation limit, detects the lower and upper range of the analyte of the interest in the formulation can be quantified with respect to precision, accuracy and linearity of the method. Whereas, detection limit of the method will identify the upper and lower range of the analyte of the interest can be identifies or detected in the formulation, but it will not meet the precision, accuracy and linearity of the method. (Qureshi *et al*., 2024; Duygu *et al*., 2017; IJzerman Boon *et al*., 2015; Vicente *et al*., 2014; Shrivastava *et al*., 2011). The robustness of an analytical method confirms that, the deliberate changes in the parameters of the method will not impacts the recovery of the analyte. These deliberate changes in the parameter of the method should meets the precision and accuracy (ICH Q14, 2023; Vicente *et al*., 2014). An objective of this study is to prove the suitability of test methods like pH, Gram's staining, spore's staining, heavy metals, arsenic, test for specific pathogens, total yeast and mold count (TYMC) and total viable count (TVC) to test the *Bacillus clausii* spores (BCS) in the form of lyophilized powder and suspensions manufactured at ELS, Hyderabad, Telangana, India for human and animal applications.

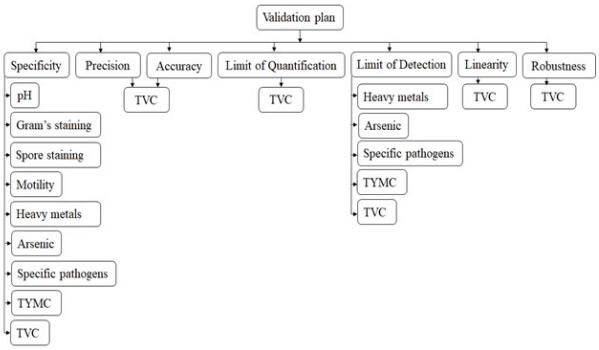

Figure 1. Method validation plan

MATERIALS AND METHODS

ELS established in-house specifications for commercial release of our *Bacillus clausii* (BC) based probiotic formulations with reference to various pharmacopeial guidelines. The specification has been classified into three main categories with identification tests, limit tests, and assay tests. These test parameters are executed for the validation parameters mentioned in Figure 1. Test samples used in this study are *Bacillus clausii* spore suspension (BCSS) with batch numbers like CSS09624, CSS09724, CSS09824, BCS00125, BCS00225 and BCS00325; BC lyophilized powder/Active pharma ingredient batch numbers like BCD01524, BCD01624, BCB00125 and BCB00225; Placebo/Purified water (PW); *Pseudomonas aeruginosa,* ATCC No.: 9027 (PA); *Escherichia coli*, ATCC No.: 8739 (EC); *Salmonella sps.,* ATCC 6017 (SS); *Staphylococcus aureus,* ATCC No.: 6538 (SA); *Aspergillus brasiliensis*, ATCC No.: 16404 (AB) and *Candida albicans*, ATCC No.: 10231 (CA).

Specificity: This parameter has been executed in three independent test runs by two different analysts on different days. Observed for the discrimination among the results of the test samples, Positive control (PC), Negative control (NC), and placebo. Specificity of the identification test parameters like pH was evaluated by analyzing the test samples along with the pH 4.0 and 9.2 buffers as per the procedure described in the USP general chapter 791, for Gram's characterization test, samples were analyzed along with the PA as per procedure described by Ahmad Paray *et al*. (2023), for spore staining test, sample were analyzed along with the PA as per modified procedure of Oktari, *et al*. (2017) and for motility test, samples were analyzed along with the SA as per procedure described by Aygan *et al*. (2007). For specificity of the assay test (Total viable count (TVC)) test, samples were analyzed along with the placebo (PW) as per the modified procedure of the USP general chapter 64. Whereas specificity of the limit tests like heavy metals and arsenic were demonstrated by analyzing the test samples along with the placebo as per the procedure described in the USP general chapters 231; 211 and EU general chapters 2.4.8; 2.4.2 respectively. The specificity of the test parameter for specific pathogens has been demonstrated by testing samples along with the standard positive cultures like EC, SS, SA, PA, and NC (Placebo) as per the procedure described in the USP general chapters 61; 62; 2021; 2022 and TYMC has been demonstrated by testing the samples along with the standard positive cultures like AB, CA and NC as per procedure described in the of USP general chapters 61; 62; 2021; 2022 and EP general chapter 2.6.12; 2.6.13.

Precision and accuracy: This validation parameter has been executed as an intra-assay precision and an intermediate assay precision. Intra-assay precision has been executed by analyzing the test samples of the single strength in a short interval of time by the single analyst in six replicates. Intermediate precision has been executed by analyzing the various strengths of the test sample from 0.5 billion (B)/5mL to 5B/5mL and 10B/g to 1500B/g) by two different analysts on three different days as per the modified procedure of the USP general chapter 64. Observed for the percent coefficient of the variation (CV%) among the results of the replicates for intra-assay precision, among the days, analysts, and strengths of the samples for the intermediate precision. For accuracy test parameter calculated the recovery of the observed results from the expected results.

Limit of detection (LOD) and quantification (LOQ): The LOD for heavy metals and arsenic has been demonstrated by diluting the standard solution (SST) lead nitrate and arsenic with placebo to various strengths like 20ppm, 15ppm, 10ppm, 5ppm, and 1ppm; 1ppm, 0.5ppm, 0.25ppm, and 0.1ppm respectively. Along with these dilutions SST and placebos

were analyzed as per the procedure described in the USP general chapters 231; 211 and EU general chapters 2.4.8; 2.4.2 respectively. Validation parameter LOD was evaluated for specific pathogens by enumerating the count of standard cultures like EC, SS, SA, PA, NC, and TYMC by enumerating the count of the standard cultures like AB, CA, and NC (Placebo) as per the procedure described in the USP general chapters 61; 62; 64; 2021; 2022 and EP general chapter 2.6.12; 2.6.13. These validation parameters like LOD and LOQ were demonstrated for the TVC test parameter by spiking the placebo with the active pharma ingredient at various strengths (0.5 billion (B)/5mL to 5B/5mL and 10B/g to 1500B/g) and analyzing the samples in three different test runs by different analysts on different days as per the modified procedure of the USP general chapter 64. Recovery has been calculated for the LOQ and CV (%) has been calculated for the LOD parameter.

Linearity and range: The coefficient of determination (R^2) has been determined for the results of test samples with the range of 0.5B/5mL to 1500B/g. Data has been retrieved from the precision, LOD, and LOQ.

An interval between the upper and lower limits of the test samples should meet validation criteria i.e. R^2 value above 0.98 indicates that the method has shown linearity between the upper and lower limits of the test sample The upper and lower limits of the test samples met the validation parameters like precision, accuracy, and linearity that upper and lower limits of the test sample indicates the range of the method. These parameters were evaluated for the assay method i.e. enumeration of TVC of the test sample.

Robustness: This validation parameter has been evaluated by analyzing the test samples by slightly modifying the method parameter, i.e., sample volume (1.050mL and 0.950mL) from the controlled condition (i.e. 1mL). All other parameters are fixed as per the methodology specified for the test parameter. Also, by modifying the method's parameter i.e. sonication time (10min 30sec and 9min 30sec) from the controlled condition (i.e. 10min), all other parameters are fixed as per methodology specified for the test parameter. The recovery has been calculated for the varied conditions from the controlled conditions. This parameter has been evaluated for the assay method i.e. enumeration of TVC of the test samples.

RESULTS

Specificity: The pH values for the test samples (Neutral) have been observed as 6.5 to 7.5, for pH buffer 4.0 (Acidic) as 4.0 ± 0.2 and for pH buffer 9.2 (Basic) as 9.2 ± 0.2. The Gram's characterization of the test samples was violet color rod-shaped bacterium with endospores and the Gram's characterization of PA was pink color rod-shaped bacteria. In the spore staining test, samples were observed as Ellipsoidal green-colored spores and PA was observed as pink color rod-shaped bacteria. The test samples were shown Brownian and SA didn't show any movement under the microscopic observation in the motility test. In the evaluation of the TVC test parameter, the expected count was observed on the petri plates, whereas for the placebo (PW) we didn't find any colonies. For heavy metals and arsenic test parameters, the color formation for the test samples was less intense than the PC (SST) and it is the same as for negative control (PW). The expected pathogens, yeast, and mold growth have not been detected for the test samples, and standard culture growth was observed while applying the same method for the analysis (Figure 2). Results of the specificity validation parameter for pH, Gram's characterization, spore staining, motility test, heavy metals, arsenic, TVC, specific pathogens, and TYMC showed clear discrimination among the results of the test samples from the standard buffers, standard cultures, SST, PC, NC and placebo.

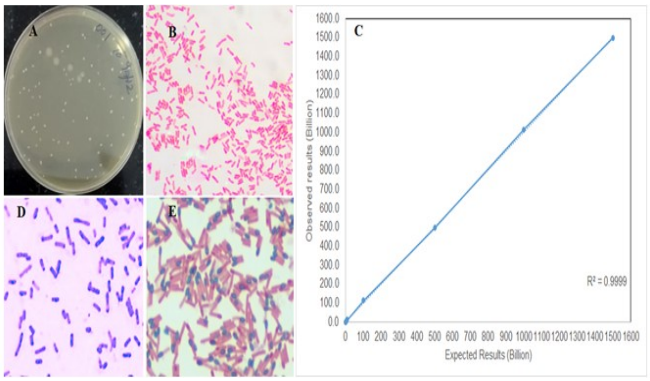

Figure 2. A. *Bacillus clausii* colony morphology on Soyabean casein digest agar, B: Gram' staining of *Pseudomonas aeruginosa*, C: Linear regression analysis, D: Gram's staining of *Bacillus clausii*, E: Spore staining of *Bacillus clausii* spore suspension

Precision and accuracy: Results of the test samples analyzed by the single analyst in a short interval of time in multiple test runs as a part of intra-assay precision the CV (%) was 2.29 to 6.20%, the variation between the results for the test samples of various strengths (0.5B/5mL to 5B/5mL and 10B/g to 1500B/g) analyzed by different analyst on different days as a part of intermediate precision the CV (%) was 0.00 to 12.37% and the recovery as a part of accuracy was 94.40 to 120.00%.

Limit of detection (LOD) and quantification (LOQ): The samples prepared by spiking with the known strength from the range of 0.5B/5mL to 5B/5mL and 10B/g to 1500B/g and analyzed in the three independent test runs have shown CV (%) of 0.00 to 10.83 and recovery of 94.40 to 120.00%.

The observed lower LOD (LLOD) and LOQ (LLOQ) values for the method is 0.5B/5mL and the upper level of the LOD (ULOD) and LOQ (ULOQ) is 1500B/g. The observed LLOD and ULOD for the heavy metals is 1ppm and 20ppm and for arsenic 1ppm and 0.25ppm. The ULOD and LLOD results for the test parameters like specific pathogens is 10^1 & 10^6 and TYMC is 10^1 & 10^5 colony forming units (CFU)/mL.

Linearity and range: Using the results of the precision, accuracy, and LOQ regression analysis were conducted using Microsoft Office Excel for the test samples of the range from 0.5B/5mL to 1500B/g, the assay method (TVC) showed more than 0.99 coefficient of determination in regression (Linearity) analysis, percent CV of 0.00 to 10.83 in precision and recovery of 94.40 to 120.00% in accuracy. These results indicate that this method has suitable linearity and a method range of 0.5B/5mL to 1500B/g.

Robustness: After deliverable changes were made in the assay parameters like sample volume and sonication time, the recovery was observed from 87.80 to 111.10% from the controlled parameter during the robustness parameter evaluation.

Table 1. Specificity results for Identification test

Test parameter	Test sample	Closely related samples	Results
pH	*B. clausii* spore suspension	pH Buffers 4.0; 9.20	Discrimination has been observed among the test samples and closely related samples
Gram's Staining		*Pseudomonas aeruginosa*	
Spore Staining		*Pseudomonas aeruginosa*	
Motility		*Staphylococcus aureus*	

Table 2. Specificity results for limit test

Test parameter	Test sample	Positive & Negative control	Observation
Heavy Metals	*B. clausii* spore suspension	Lead nitrate (20ppm) & Placebo	Discrimination has been observed among the test samples, Positive and Negative control
Arsenic		Arsenic (1ppm) & Placebo	
Test for Specific Pathogens		*Pseudomonas aeruginosa; Staphylococcus aureus; Escherichia coli; Salmonella sps.* and Placebo	
Total Yeast and Mold Count		*Candida albicans; A. brasiliensis* and Placebo	

Table 3. Results of validation parameters for assay test

Sample strength (Billion)/ Validation parameters	Specificity	Intra-assay precision	Inter-assay precision	Accuracy	Linearity	LOD	LOQ	Range	Robustness
Limits as per ICH Q2R2	Method should be Specific	CV (%): <20%	CV (%): <20%	Recovery (%): 70 – 130%	R^2: >0.98	CV (%): <20%	Recovery (%): 70 – 130%	R^2: >0.98	Recovery (%): 70 – 130%
0.5 billion CFU/5mL	*B. clausii* growth has not been observed in the placebo	CV (%): 2.29 to 6.20%	CV (%) for Day to Day: 0.69 to 12.37% CV (%) for Analyst to Analyst: 0.00 to 5.44%	Recovery is (%): 94.40 to 120.00%	Correlation coefficient is >0.99	CV (%): 0.00 to 10.83%	Recovery is (%): 94.40 to 120.00%	Correlation coefficient is >0.98	Recovery is (%): 87.80 to 111.10%
1.0 billion CFU/5mL									
1.5 billion CFU/5mL									
2.0 billion CFU/5mL									
2.5 billion CFU/5mL									
3.0 billion CFU/5mL									
4.0 billion CFU/5mL									
5.0 billion CFU/5mL									
10 billion CFU/g									
100 billion CFU/g									
500 billion CFU/g									
1000 billion CFU/g									
1500 billion CFU/g									

Table 4. Limit of detection (LOD) and quantification (LOQ) results for limit test

Test parameter	Test sample	Observations
Heavy Metals	Lead nitrate (20ppm, 15ppm, 10ppm, 5ppm & 1ppm) & Placebo	Lower and upper limit of detections are 1ppm & 20ppm respectively.
Arsenic	Lead nitrate (1.00ppm, 0.50ppm, 0.25ppm, & 0.10ppm) & Placebo	Lower and upper limit of detections are 0.25ppm & 1ppm respectively.
Test for Specific Pathogens	Standard cultures like *Pseudomonas aeruginosa* (23×10^6); *Staphylococcus aureus* (16×10^6); *Escherichia coli* (12×10^6); *Salmonella sps* (18×10^6)	Lower and upper limit of detections are 10^{-6} dilution & 10^{-1} respectively.
Total Yeast and Mold Count	Standard cultures like *Candida albicans* (18×10^5); *A. brasiliensis* (27×10^5)	Lower and upper limit of detections are 10^{-5} dilution & 10^{-1} respectively.

DISCUSSION

To ensure the quality and safety of the probiotic, as per the recommendations of the regulatory agencies and customers ELS established specifications with Identification tests (pH, Gram's, spores staining and motility tests), Assay tests (TVC) and limit tests (Heavy metals, arsenic, specific pathogens, and TYMC). To prove the suitability of the methods all the test methods were validated for specified parameters as mentioned in Figure 1. The results of the pH test parameter clearly show the discrimination among samples and buffers with different pH values, Gram's characterization method has shown the discrimination among the gram-positive and gram-negative, spore staining method among spores and vegetative cells of the bacteria and motility test among the motile and non-motile bacteria. The viable count of the BC was not impacted due to the presence and absence of the components expected to be present; it was proven by comparing the results of the test samples with placebo and recovery studies. The limit tests of the test samples also showed a clear difference between the PC and NC. According to the ICH Q2R2 guidelines, the method should discriminate the results of the test samples from the closely related compounds, recovery should not be impacted by the presence or absence of the components expected to be present and differences should be observed with the PC and NC. All the results of the test parameters are within the criteria specified by the authority and concludes that these parameters show the specificity of the method for analysis of samples. The precision parameter showed the results of 0.00 to 12.37% at the various strengths of the sample analyzed in multiple assays runs in a short period, analyzed by different analysts on different days. As per the ICHQ2R2 and USP, the CV (%) should not be more than 20%, whereas our method has a maximum of 12.37 CV (%) only. It represents that the modified method of USP <64> for the enumeration of TVC for the test samples has suitable precision over the range of the method. The accuracy and LOQ parameters showed the results of 94.40 to 120.00% recovery from the expected results among the range of the method at various strengths of the sample analyzed by different analysts on different days. As per the ICHQ2R2 and USP, the recovery should be 70-130%. It represents that the modified method of USP general chapter 64 for the enumeration of TVC has suitable accuracy over the range of the method. The lower limit of detection for the heavy metals is 1ppm, arsenic is 0.25ppm, specific pathogens is 10^6, TYMC is 10^5 and TVC is 10^8 cfu/mL, whereas, the upper limit of detection for the heavy metals is 20ppm, arsenic is 1ppm, specific pathogens is 10^1, TYMC is 10^1 and TVC is 10^6 cfu/mL. According to the ICH and USP guidelines the specification of the test parameter should be in-between the ULOD and LLOD. Even after deliverable changes were made in the assay parameter also the results were not impacted, it indicates that the parameters are not critical parameters for the analysis of the test samples for TVC. According to ICH and USP after deliverable changes are made in the parameters of the method results should not be impacted.

The test samples from the range 0.5B/5mL to 1500B/g analyzed as per the modified USP general chapter 64, all the test samples showed more than 0.98 coefficient of determination in regression (Linearity) analysis, CV (%) of 0.00 to 12.37 in precision and recovery of 94.40 to 120.00% in accuracy. According to the ICHQ2R2, USP and FDA methods should have an R^2 value of not less than 0.98, a precision of not more than 20% CV (%), and an accuracy of 70-130%. All the strengths of the test samples from 0.5B/5mL to 1500B/g met the validation criteria concerning linearity, precision, and accuracy and concluded that the method range is 0.5B/5mL to 1500B/g. These results indicate that this method has suitable linearity and a method range of 0.5B/5mL to 1500B/g.

CONCLUSION

As per the results and discussions, all the test methods adopted, modified from the monograph and literature met validation criteria and these methods are suitable for the intended purpose of use i.e release the commercial test samples.

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CONFLICT OF INTEREST STATEMENT: The authors declare that there are no competing interests.

ETHICAL APPROVAL: Animals were not used for the execution of this research work.

AUTHORS AND CONTRIBUTIONS

All the experiments were designed and carried out and the manuscript was prepared by SB with the help of RM, PT, SA, NB, SP and DG. All authors read and approved the final manuscript.

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