DEVELOPMENT OF VALIDATED QUANTITATIVE REFERENCE MATERIALS FOR APPLICATION IN FOOD MICROBIOLOGICAL TESTING

Thesis Submitted for the Award of the Degree of

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in

Microbiology

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2025

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Development of Validated Quantitative Reference Materials for Application in Food Microbiological Testing**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision Dr. Rohit Rai, working as Assistant Professor, in the Faculty of Applied Medical Science of Lovely Professional University, Punjab, India and co-supervision of Prof. (Dr.) Santhini S. Nair, working as Vice Principal, Head of Department Microbiology & Associate Professor in the Vivekanand Education Society's College of Arts, Science & Commerce. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **Development of Validated Quantitative Reference Materials for Application in Food Microbiological Testing**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Microbiology/ School of Bioengineering and Biosciences , is a research work carried out by Kumud Ashish Singh, 42000343, is bonafide record of ~~his~~/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Evolving microbiological testing methods have resulted in the imperative need for the development of different types of microbiological reference materials (RM). Research articles available in this area reveals that development in RM has been quite substantial in chemical testing as compared to microbiological testing, the primary reasons being stated, as the ease of spiking and recovery in chemical RM when compared to microbiological RM. A major challenge in achieving recovery and stability during the development of quantitative microbiological reference materials is influenced by factors such as temperature, the type of microbiological media utilized, the analysis method, interference from antimicrobial agents in food matrices, and the presence of competitor microorganisms in greater quantities than the target microorganisms. Most of the research papers in microbiological RM development are from developed economies with limited literature available for complex food matrices. An analysis of different International Depository Agencies under the Budapest treaty revealed that there are only three institutes based in Europe that provide quantitative or assigned value RM in Microbiology, thereby highlighting the scarcity in the availability of quantitative RM in Microbiology. RM plays a critical role in addressing data accuracy situations and the usage of the same has been mandated by ISO 17025:2017 for accredited laboratories. Presently in Microbiology food testing laboratories qualitative RM rather than quantitative RM is used due to the ease of availability and lucrative cost point of the former.

The present study was conducted with three objectives of finding the best possible combination of nutritive and protective agents to be incorporated in the development and production of viable and stable assigned value or quantitative microbiological RM along with the creation of PT samples with the most common food matrices (skimmed milk powder) and validation with commercially available quantitative RM. Nutritive agents such as casein hydrolysate, soy protein hydrolysate, and fish protein hydrolysate at the concentrations of 5, 10, 15, 20 and 25% were evaluated for long term survival of two test microorganisms, *Escherichia coli* MTCC 1610^{T}& *Salmonella enterica* subspecies

Arizonae MTCC 660^T. The effect of each of the hydrolysates was individually studied on *E. coli* & *S. enterica* at two different storage temperatures (5±2°C and -18±3°C). Response surface methodology (RSM) was used for the optimization of the experimental run. Soy protein hydrolysate and fish protein hydrolysate (mentioned concentrations) resulted in the long-term survival of *E. coli*. However, when agar was introduced as growth media during inoculum preparation. *E. coli* showed enhanced resistance and long-term survival even in the presence of 5 – 15% fish protein hydrolysate. Fish protein hydrolysate was found as the only effective nutritive agent for *S. enterica*. All three hydrolysates were found to be ineffective in the survival of *E. coli* & *S. enterica* at -18±3°C, as they were incapable of imparting cryoprotection. Hence for long term survival, cryoprotectants were incorporated and assessed with RSM optimization. Glucose, glycerol, trehalose, and bovine serum albumin were selected as the protective agents to be used in the formulation. The final stage in the development of assigned value reference material involved encapsulation using 2% carboxy methyl cellulose and 3% gelatin, so as to reduce water activity and to ensure that the developed product will have water solubility. The produced reference materials were then assessed for homogeneity, stability, and extended shelf life.

One way ANOVA was applied on the viability data of the produced quantitative RM of *S. enterica* and F_{stat} (0.03) $<F_{critical}$(3.89), p-value (0.97) > 0.05 with overall mean log variation of the counts between the groups being (0.03) ≤ 0.1 leading to acceptability of homogeneity. The standard deviation observed was 0.14 ≤ Log_{10} 0.35 at 4 months. The developed assigned value RM of *S.enterica* had viability up to 5 months and was homogeneous and stable at -18±3°C for 4 months. Similarly, a one-way ANOVA was applied to the viability data of the produced quantitative RM of E. coli. The results showed that F_{stat} (0.08) < $F_{critical}$ (3.68), and the p-value (0.92) > 0.05, with an overall mean log variation of the counts between the groups being 0.12 ≤ 0.1, indicating the acceptability of homogeneity. The standard deviation observed was log_{10} 0.17 ≤ Log_{10} 0.35 and log_{10} 0.33≤ Log_{10} 0.35 at 4 and 5 months, respectively. The developed assigned

value RM of *E. coli* had viability up to 5 months and was homogeneous and stable for 4 months at -18±3°C .

The quantitative RM thus produced was subjected to inclusion of skimmed milk powder (SMP) as food matrix to create a proficiency testing (PT) sample. Direct inclusion of SMP in the produced quantitative RM resulted in contamination, low recovery, and loss in viability. Hence it was decided to prepare 10% sterile SMP separately and then inoculate in the produced quantitative RM before studying the inclusion of food matrix. Ten samples of produced quantitative RM of each microorganism were checked, and the z score was found to be within ± 1 indicating a normal distribution bell curve. Chi square was calculated for the observed and expected value which was observed to be insignificant to the Chi square critical value thus accepting the null hypothesis that there is homogeneity and appropriate recovery even with inclusion of SMP in the produced quantitative RM. Chi square calculated for *E. coli* was 0.04 and for *S. enterica* as 0.06, which is < than P value of 16.92 at 0.05 significance and 9 degrees of freedom.

The produced quantitative RM was validated at five different laboratories which include three commercial laboratories and two academic laboratories. The z-score calculated from the laboratory reported results for *E. coli* and *S. enterica* was within ± 1 indicating normal distribution bell curve and the standard deviation of all the reported results was 0.07 for both the microorganisms indicating no significant deviation in the reported results of the laboratories. Ten samples of the commercially available quantitative RM showed a 0.21 standard deviation which was at par with the produced quantitative RM. Hence, we were able to develop homogeneous RM of *E. coli* and *S. enterica* with a shelf life of 4 months. This study can be extended further to increase the shelf life by working on reduction of water activity, inclusion of other protective agents along with exploring lower temperature storage of – 80°C.

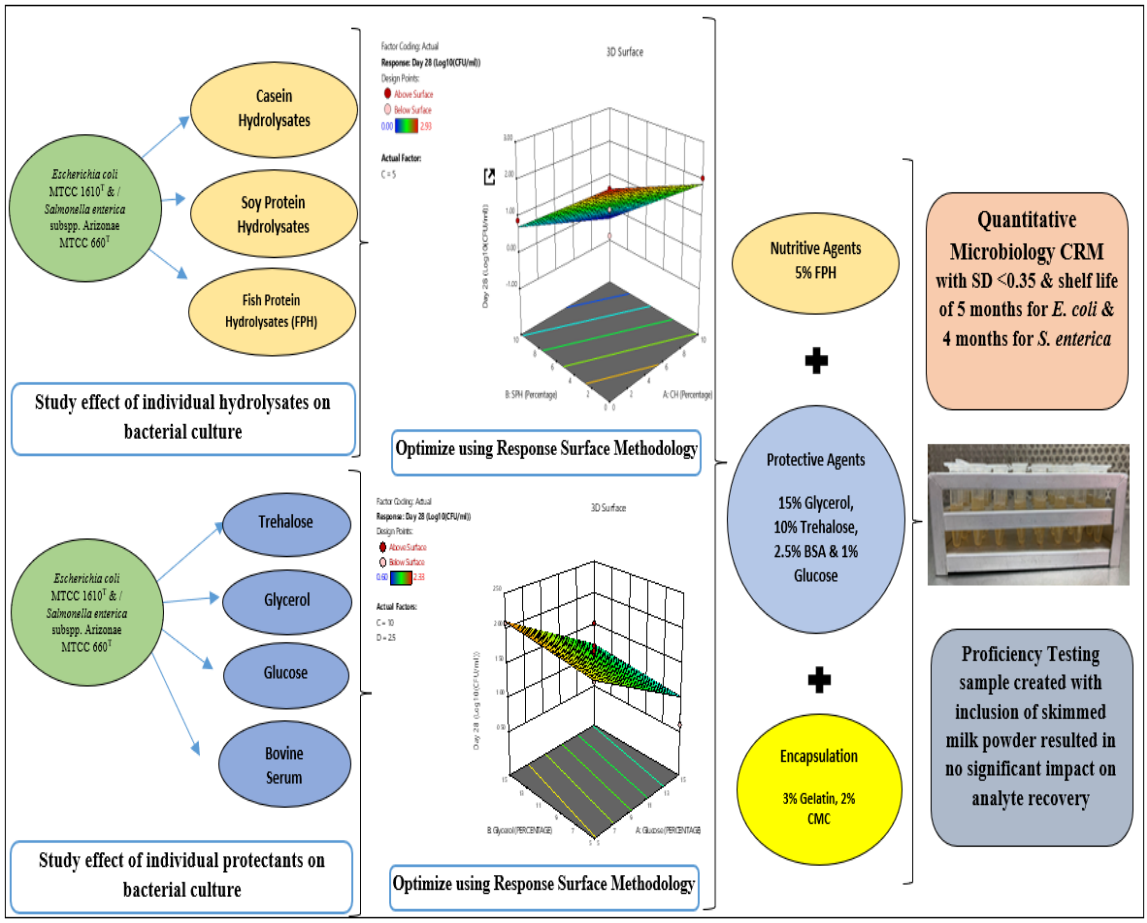

Figure A1. Graphical abstract summarizing research work & key findings

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Date: Kumud Ashish Singh

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CHAPTER 1

INTRODUCTION

1.0 Introduction

Reference materials (RM) are used in measurement processes like analytical testing and calibration to ensure data or measurement accuracy. RM are the known value samples that analysts use to understand whether the test method in use is giving appropriate results or fit for purpose. ISO 17025:2017, which is an accreditation standard for testing and calibration laboratories, has popularized the usage of RM in food testing laboratories, wherein RM are used in all the stages of the measurement process (1). For example, RM are being used for method validation, calibration, quality control, interlaboratory comparisons for method validation, method verification, including regular monitoring of method performance in the laboratory and for assessing laboratory proficiency (2,3). RM are essential tools for quality control of any type of testing or analysis (4). The reliability of RM depends on various properties such as stability, homogeneity and suitability or fitness to its purpose (5). Stability, homogeneity, and recovery are critical and challenging factors in the development of microbiological RM (6). Based on the available format for microbiological testing, RM can be broadly classified as (i) Qualitative RM; (ii) Quantitative RM; (iii) when (i) and (ii) have incorporated food matrices; (iv) Molecular RM, e.g., genomic nuclear material (like DNA); (v) Toxins produced by microorganisms (like mycotoxin, enterotoxin) (7).

In routine testing, samples are unknown in terms of their expected value; hence RM are used to confirm that all the processes involved in testing are leading to accurate results. When RM has assigned or certified values, they are referred to as certified reference materials (CRM).

According to the definition given by the International Organization of Standards (ISO) (2), RM are materials that possess sufficient homogeneity and stability for one or more specified properties. These properties have been established and are suitable for their intended use in measurement analysis. A certified reference material is defined as a reference material that is characterized by a metrologically valid procedure. This means it is traceable to an unbroken chain of metrological traceability, which leads back to the

respective metrological institutes within one's own country or another country. This traceability applies to one or more specified properties. As per standards (1), accredited microbiology laboratories need to use certified reference materials (CRM) to achieve measurement accuracy. For any kind of testing that generates measurement values, either qualitative or quantitative, accuracy is the most important requirement, without which the entire effort of the analysts goes in vain (8). The measurement process varies in nature, considering the evolution of testing methodologies from as simple as measuring temperature to as complex as determining emerging contaminants in food. One of the significant differences between microbiological and chemical analysis is the involvement of living organisms as an analyte in the former type of testing. Testing methods in microbiology can be categorized into qualitative (detection, presence or absence, identification of microorganisms, etc.) and quantitative (number of colonies or colony-forming unit(s) per sample) methods based on the quantity of sample size (9). The size of the sample can vary from 1 gram to 25 grams to 375 grams, and in some cases up to 750 grams which may be defined by the regulatory bodies or testing method. The typical microbiological testing involves sample preparation (which can include sample weighing, homogenization, serial dilution using diluents), enrichment (pre-enrichment for resuscitation and /or selective enrichment for amplifying selective microorganisms of interest), culturing (subculture from enrichment broth to solid media) and biochemical identification involving incubation at various stages of testing. As per ISO standard (10), accuracy refers to both trueness and precision. It can be put forward in the simple equation as follows:

Accuracy = Trueness + Precision.

Based on this, accuracy can be defined as the closeness of results with each other (in case of more than one reading) and the true or reference value. When an actual true value is unknown, a mutually agreed or observed reference value is accepted. Trueness is defined as the closest agreement between the obtained average value and accepted reference value (ISO 5725-1:1994). The accepted reference value (ISO 5725-1:1994) is "a

value that serves as an agreed-upon reference for comparison, and is derived as one of the following: a) theoretical or assigned value based on scientific principles,

b) an assigned or certified value based on experimental work by a national or international organization,

c) a consensus or certified value based on collaborative experimental work under the auspices of a scientific group,

d) when a), b) and c) or not available, the expectation of the measurable quantity, i.e., the mean of a specific population of measurements." In certain cases where outliers are observed or expected, the mean is replaced by a median, or simply, outliers are not considered for deriving the mean.

Commonly available CRM for microbiological laboratories are primarily qualitative and limited to strain traceability to culture collection as compared to chemical laboratories, where CRM have certified value as well. There are only three international depositories across the globe that provide microbiological quantitative RM. Hence, to improve measurement accuracy in microbiological laboratories, a focus on the usage of quantitative RM is required for estimating the accuracy and precision of quantitative microbiological methods. To achieve this, certified or assigned value microbiological RM should be cost-effective and readily available indigenously within the country.

Another microbiological RM format, which is used for the Proficiency Testing Program, includes food matrices and assigned value target microbial strains and /or competitor microorganisms. RM in food matrices with competitor microorganism/s are used in the proficiency testing program (2). The Centers of Disease Control and Preventions (CDC) has published competency guidelines for public health laboratory professionals, which mentions participation in proficiency testing (PT) as a part of regulatory compliance for public health laboratories in the microbiology testing domain (11). Microbiology testing is considered critical for public health as it helps in detecting and identifying outbreaks, new diseases, and biological threats. One of the objectives of

National Reference Laboratories (NRLs) established by the German Federal Institute for Risk Assessment (BfR) is to conduct inter-laboratory studies and participate in ring trials and proficiency tests within the national control program for *Salmonella* (12). Participation in PT to assess analytical skills is mandatory for all laboratories accredited under ISO 17025:2017. Furthermore, as per ISO 17043:2023, PT evaluates the performance of participants or laboratory analysts against pre-established criteria through interlaboratory comparisons. PT is also called external quality assessment (EQA) in the clinical or medical field, as it is an assessment of the analytical performance of an indigenous laboratory with its contemporaries or to an accuracy-based reference system (13). The frequency of participation in the PT scheme is dependent on guidance documents released by the International Laboratory Accreditation Cooperation (ILAC), local geographical accreditation committees (Asia Pacific Accreditation Cooperation, European Accreditation), and the accreditation bodies responsible for that country. Hence, PT frequency can be annual or biannual for all testing parameters or sometimes can be dependent on the laboratories to choose, based on their assessment (14). There are commercial PT providers (PTPs) across the globe, primarily situated in the USA or EU, who provide PT samples, and most of the developing countries in Asia import PT samples from them (15). Despite the scarcity of literature on the creation of PT samples, only a few countries in Asia, namely China and Vietnam, have in-house manufacturers of microbiological PT samples accredited under ISO 17043:2023.

This study focuses on the development of quantitative microbiological RM, PT samples with food matrix (skimmed milk powder) and validation of the produced RM along with commercially available RM. Further, the development of quantitative microbiological RM by using commonly used preservation techniques, identifying appropriate nutritive and protective agents along with determination of homogeneity, stability, and assigned value calculation of the developed quantitative RM will be emphasized in this study. After the development of quantitative microbiological RM, skimmed milk powder can be included as a food matrix to create PT samples. The selected target analytes or microorganisms for the development of quantitative RM and PT samples

are *Escherichia coli* and *Salmonella enterica*. These two are among the most significant foodborne pathogens implicated in various food safety-related recalls. The performance of the developed quantitative RM, in terms of homogeneity, stability, and shelf-life, will be evaluated in comparison with commercially available quantitative RM.

CHAPTER 2

REVIEW OF LITERATURE

Published as

Singh et al. (2022) "Review on development of assigned value microbiological reference materials used in food testing" Doi: 10.1016/j.fm.2021.103904

Singh et al. (2023) "Survival of *Salmonella spp.* and Pathogenic *Escherichia coli* in Food Matrixes and Its Relevance in the Development of Proficiency Testing Samples" Doi: 10.1093/jaoacint/qsad011

2.0 Review of Literature

Reference materials are widely used in chemical and biological testing laboratories to ensure data accuracy as per the requirements of ISO 17025:2017 (1). Microbiological qualitative reference materials (RM) offer traceability to culture collections and are widely used due to their availability and cost-effectiveness. However, qualitative RM only allows for a basic assessment of performance, as they indicate merely the presence or absence of growth. In contrast, quantitative or assigned value RM has great benefits compared to qualitative RM. Quantitative RM helps in data accuracy and can support troubleshooting by identifying the root cause. It has been highlighted that the scarcity in availability and high cost of quantitative microbiological RM is due to fewer numbers of RM manufacturers (16).

2.1 Application and Availability of Microbiological Reference Materials based on Global and Indian Scenario

In developed economies like Europe (EU) and the United States (USA), certified value RM (CRM) is more emphasized and used in laboratories during routine testing (17). CRM plays a significant role in ensuring the quality of data produced in testing laboratories. Quantitative RM is used for method validation, quality control, instrument calibration, research and development and standardization of process (18,19). The amount of research available for microbiological certified value reference material is 1.3% (approximately) compared to 87.5% (approximately) for chemical and related certified reference material in the Scopus database. Table 1 provides the list of institutes under the Budapest Treaty that also act as international depository agencies (IDA) and support researchers by providing different types of microbiological RM required for food testing (20). Forty-eight institutes across twenty-six countries have been identified as IDA, which act as depositories for different types of microbiological RM. These IDA institutes were further assessed by visiting their websites to acquire knowledge on the type of microbiological RM provided as part of their services. Hence, it can be concluded that there has not been enough study available on the development of assigned or certified value reference material in

microbiology. Further, on analyzing the data from Table 1, it can be concluded that though we have thirty-nine institutes across twenty-six countries in the IDA list providing qualitative RM for microbiology food testing, the number of institutes that provide quantitative RM for microbiology is as less as three institutes across three countries, i.e., France, Germany, and the UK. There are thirty institutes across twenty-one countries that provide RM for molecular analysis. At the same time, only two institutes in two countries (Australia & USA) provide microbial toxins RM. One of the reasons for the low number of institutes providing microbial toxins RM may be the chemical nature of microbial toxins, which effectively pushes them under the chemical RM category rather than the microbiological RM. Since only three institutes provide quantitative RM and all of them are in Europe, there is an urgent need for growing economies like India to develop quantitative or assigned value microbiological RM. This will help growing economies to attain data accuracy and have regulatory compliance for microbiological criteria that have been established by regulatory agencies like the Food Safety Standards Authority of India (FSSAI) (21). A microbiological criterion for food as per the definition in FAO (Food and Agricultural Organization) is "the acceptability of a product or a food lot, based on the absence or presence, or the number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot." (22). Microbiological criteria can be further categorized as food safety microbiological criteria or quality microbiological criteria. As per CODEX, microbiological criteria have been used for many years (23). Microbiological criteria contribute to the improvement of food hygiene, even though they are based on empirical observation achieved by existing measures without any direct link to ALOP (appropriate level of protection) concerning public health (24). FSSAI has also established microbiological criteria for various food items, and one of its essential tasks is the surveillance of food produced in India, for which there are various FSSAI recognized microbiology food testing laboratories that assess the microbiological criteria. The food business operators who are unable to meet the FSSAI established microbiological criterion or criteria are subjected to legislative proceedings, which could lead to penalties, business loss, and confinement of business owners in some cases. In order to ensure the

accuracy of the testing procedures conducted by FSSAI recognised laboratories, it is imperative that these laboratories are accredited to ISO (1).

Institute of Microbial Technology (IMTech) and National Chemical Laboratory (NCL) provide microbiological CRM where the strain is traceable to an internationally recognized culture collection in India. National Physical Laboratory (NPL) is the national metrological institute (NMI) or national measurement institute of India like the National Institute of Standards and Technology (NIST) in the USA or the Institute of Reference Material and Measurement (IRMM) in the EU. NPL has all RM under its wing except microbiological reference material. From 2017, NPL has come up with BND™ (Bharatiya Nirdeshak Dravya) as a trademark for RM produced in India. NPL encourages RM manufacturers and start-ups to produce reference material in India. Production of good quality RM in India has now been recognized as an essential step by NPL. Apart from the institutes listed in Table 1, various RM manufacturers worldwide provide assigned value RM for microbiological analysis. Table 2 provides a list of key commercial organizations manufacturing quantitative microbiological RM, along with the country of location for manufacturing operations. Some of the different formats of quantitative microbiological RM without the inclusion of food matrix as listed in the Table 2 includes dried discs (Lenticules® and Vitroids™), freeze-dried (BioBalls®), and lyophilized pellets (Epower™, easi-tab™). Figure 1 provides details of few key institutes providing different formats or types of RM. The formats listed over here or in figure 1 are not exhaustive; a considerable amount of work is required to develop better formats. Another microbiological RM format includes food matrices and assigned value target microbial strains and or competitor microorganisms. Competitor microorganisms could be from the same Family or similar Gram staining nature (e.g., presence of *Enterobacter* with *E. coli*) or the commonly present non-pathogenic microorganisms in a particular food matrix (e.g., *Lactobacillus* in curd). RM in food matrices with competitor microorganism/s is used in the Proficiency Testing Program (2). With the advances in technologies and the increased usage of techniques like polymerase chain reaction (PCR), there is a growing need for nuclear material RM in microbiology. Similarly, toxins produced by food pathogens such

as *Staphylococcus aureus*, or mycotoxins produced by molds like *Aspergillus niger*, have resulted in the development of purified toxin RM as well (25,26).

There are different formats available for RM in microbiology, like quantitative or qualitative viable cells, combined in different food matrices like milk powders, cheese, etc., and nuclear materials, toxins produced by pathogenic food bacteria. The essential points to consider in the development of microbiological RM are homogeneity and stability at different temperatures, including storage and transportation. Different media, methods of analysis, interfering agents, and complex food matrices can also impact the recovery of microorganisms in reference material. The focus of our study is quantitative RM of foodborne pathogens, and the scientific literature available for the same has been elaborated in the next section

Table 1: Key IDA institutes offering microbiological reference materials for food testing*

Country	International Depositary Authority (IDA)	Types of RM	Reference
Australia	The National Measurement Institute (NMI)	Qualitative RM, Molecular – Genomic RM, Toxins	http://www.measurement.gov.au
Belgium	Belgian Coordinated Collections of Microorganisms (BCCM)	Qualitative RM, Molecular – Genomic RM	http://bccm.belspo.be/index.php
Bulgaria	National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC)	Qualitative RM, Molecular – Genomic RM	http://www.nbimcc.org
Canada	International Depository Authority of Canada (IDAC)	Qualitative RM, Molecular – Genomic RM	https://www.canada.ca/fr/sante-publique/programmes/autorite-depot-internationale-canada.html
Chile	Colección Chilena de Recursos Genéticos Microbianos (CChRGM)	Qualitative RM, Molecular – Genomic RM	www.cchrgm.cl
China	China General Microbiological Culture Collection Center (CGMCC)	Qualitative RM, Molecular – Genomic RM	http://www.cgmcc.net
	Guangdong Microbial Culture Collection Center (GDMCC)		http://www.gimcc.net
	China Center for Type Culture Collection (CCTCC)		http://www.cctcc.org.cn
Czech Republic	Czech Collection of Microorganisms (CCM)	Qualitative RM, Molecular – Genomic RM	www.sci.muni.cz/ccm

Finland	VTT Culture Collection (VTTCC)	Qualitative RM	http://culturecollection.vtt.fi
France	Collection nationale de cultures de micro-organismes (CNCM)	Qualitative RM, Quantitative RM, Molecular – Genomic RM	https://www.pasteur.fr/en/public-health/biobanks-and-collections/collection-nationale-cultures-microorganismes-cncm
Germany	Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)	Qualitative RM, Quantitative RM, Molecular – Genomic RM	http://www.dsmz.de/
Hungary	National Collection of Agricultural and Industrial Microorganisms (NCAIM)	Qualitative RM	http://ncaim.etk.szie.hu
India	Microbial Type Culture Collection and Gene Bank (MTCC)	Qualitative RM, Molecular – Genomic RM	https://mtccindia.res.in
	Microbial Culture Collection (MCC)		http://www.nccs.res.in
	National Agriculturally Important Microbial Culture Collection (NAIMCC)		http://www.nbaim.org.in
Italy	Collection of Industrial Yeasts DBVPG	Qualitative RM, Molecular – Genomic RM	http://www. dbvpg.unipg.it
	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna		https://www.izsler.it
Japan	National Institute of Technology and Evaluation (NITE), NBRC	Qualitative RM, Molecular – Genomic RM	http://www.nite.go.jp/en/nbrc/patent/npmd/index.html
Latvia	Microbial Strain Collection of Latvia (MSCL)	Qualitative RM, Molecular – Genomic RM	http://mikro.daba.lv

Mexico	Colección de Microorganismos del CNRG (CM-CNRG)	Qualitative RM, Molecular – Genomic RM	http://cmcnrg.inifap.gob.mx/servicios.html
Morocco	Collections Coordonnées Marocaines de Microorganismes (CCMM)	Qualitative RM	www.ccmm.ma
Netherlands	Westerdijk Fungal Biodiversity Institute (CBS)	Qualitative RM, Molecular – Genomic RM	http://www.westerdijkinstitute.nl
Poland	IAFB Collection of Industrial Microorganisms Institute of Agricultural and Food Biotechnology (IAFB)	Qualitative RM	https://www.gbif.org/
	Polish Collection of Microorganisms (PCM)		www.pcm.org.pl
Republic of Korea	Korean Agricultural Culture Collection (KACC)	Qualitative RM, Molecular – Genomic RM	http://www.genebank.go.kr
	Korean Collection for Type Cultures (KCTC)		http://kctc.kribb.re.kr/
	Korean Culture Center of Microorganisms (KCCM)		http://www.kccm.or.kr/
	Korean Cell Line Research Foundation (KCLRF)		http://cellbank.snu.ac.kr
Russian Federation	Russian National Collection of Industrial Microorganisms (VKPM)	Qualitative RM, Molecular – Genomic RM	http://www.vkm.ru/collecti.htm
	Russian Collection of Microorganisms (VKM)		http://www.vkm.ru
Slovakia	Culture Collection of Yeasts (CCY)	Qualitative RM (yeast only)	http://www.ccy.sk/

Spain	Colección Española de Cultivos Tipo (CECT)	Qualitative RM, Molecular – Genomic RM	http://www.cect.org
Switzerland	Culture Collection of Switzerland (CCOS)	Qualitative RM, Molecular – Genomic RM	http://www.ccos.ch
United Kingdom	CABI Bioscience, UK Centre (IMI)	Qualitative RM, Quantitative RM**, Molecular – Genomic RM	http://www.cabi.org
	National Collection of Type Cultures (NCTC)		http://www.phe-culturecollections.org.uk
	National Collection of Yeast Cultures (NCYC)		http://www.ncyc.co.uk
	European Collection of Cell Cultures (ECACC)		http://www.ncimb.com
	National Collections of Industrial, Food and Marine Bacteria (NCIMB)		
United States of America	Agricultural Research Service Culture Collection (NRRL)	Qualitative RM, Molecular – Genomic RM, Toxins	http://nrrl.ncaur.usda.gov
	American Type Culture Collection (ATCC)		http://www.atcc.org
	Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) at Bigelow Laboratory for Ocean Sciences		https://ncma.bigelow.org

*Content in this table has been extracted from global list of institutes as per the Budapest treaty

**Quantitative RM is provided only by NCTC (Public Health England, UK

Table 2: List of key commercial organizations manufacturing quantitative microbiological reference materials

Organization Name	Trade Name	Manufacturing Location	Source
Biosisto	-	Netherlands	https://www.biosisto.com/
Biomerieux	BioBall®	Australia	https://www.biomerieux-industry.com/
FAPAS	-	UK	https://fapas.com/
ielab	BACredi	Spain	https://www.ielab.es/en/
Livsmedelsverket	-	Sweden	https://www.livsmedelsverket.se/en
LGC	easi-tab™	UK, USA	https://www.lgcstandards.com/
MilliporeSigma	Lenticule® Vitroids™	Switzerland	https://www.sigmaaldrich.com/
Microbiologics	Epower™	USA	https://www.microbiologics.com/
NSI Lab Solutions	Microgel-Flash™	USA	https://www.nsilabsolutions.com/
	Snap-Stick™		
	Unit Dose™		
FAPAS	-	UK	https://fapas.com/

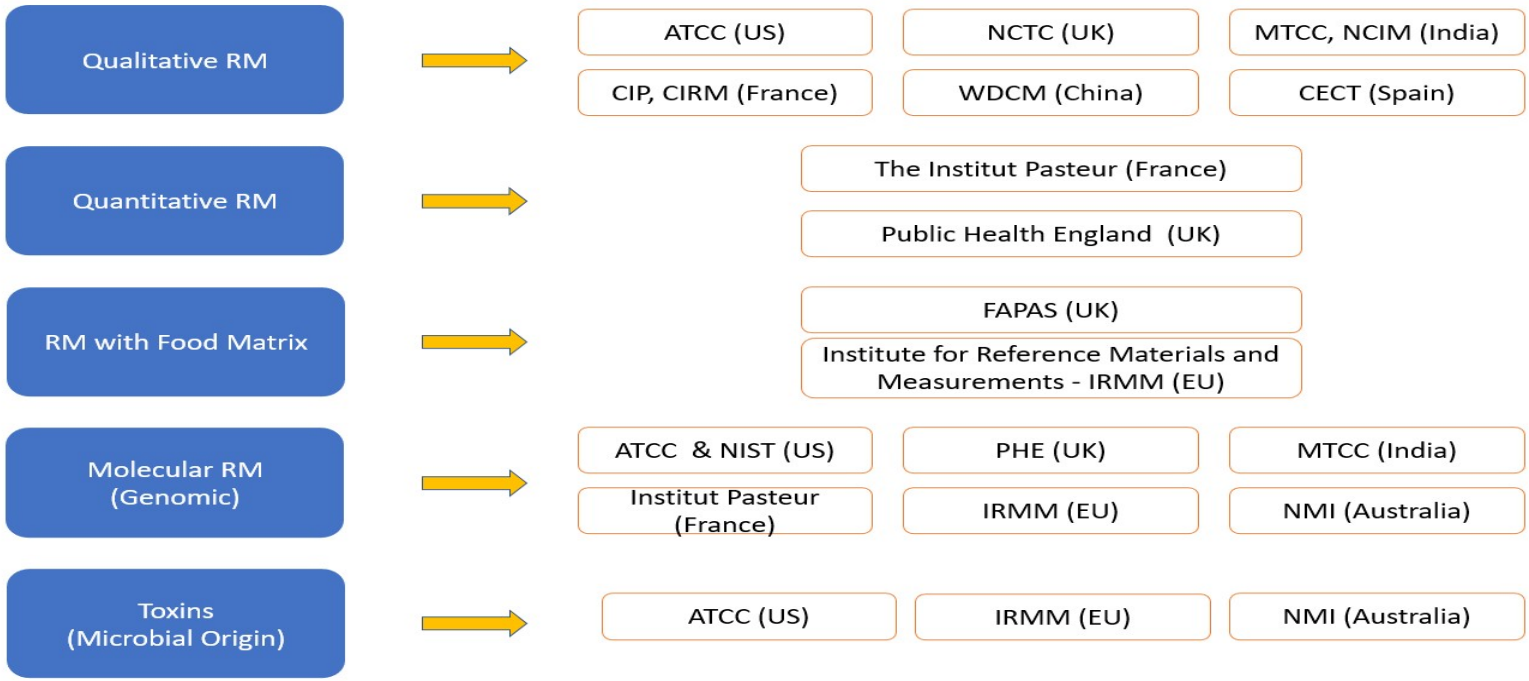


Figure 1. List of few key institutes providing different types of microbiological reference materials

2.2 Quantitative RM

Quantitative RM of *Bacillus cereus* (ATCC 9139) was prepared by incorporating its spores in milk powder matrix (27).The purity of *Bacillus cereus* (ATCC 9139) culture was confirmed on sheep blood agar (SBA) which was incubated at 37±1°C for 24h. A single colony was selected and was suspended in peptone saline (PS) solution which was 8further used to inoculate polymyxin pyruvate egg yolk bromothymol blue agar (PEMBA) for rapid sporulation of *B. cereus*. After incubation, PS solution was used to harvest *B. cereus* sporulated cells. The suspension was collected and heated to inactivate vegetative cells. Thus, the heated suspension containing only spores was added to sterilized milk and termed as high contaminated milk powder (HCMP). To obtain the desired number of colony-forming particles (cfp) in the reconstituted capsule solution, 0.014% of HCMP was mixed with commercially available sterile skimmed milk powder (SMP) which was initially gamma-irradiated and spray-dried. The mixing of HCMP with skim milk powder was done in multiple steps, wherein at each step, equal amounts of contaminated milk powder were mixed with SMP. In the final step, 4 kg of highly contaminated milk powder (HCMP) was mixed with approximately 3 kg of sterile SMP. To ensure proper mixing, a mortar and pestle were used, followed by spreading the mixture with a paper card. This process was repeated three times to obtain a homogeneous sample. A mixing apparatus (Bachofen, Basel, Switzerland) was used later to obtain a homogeneously mixed powder. Gelatin capsules, pre-sterilized by gamma irradiation, were filled with the mixed powder using an aluminium apparatus in a laminar airflow cabinet. Each of the 60 capsules contained 0.317 grams of powder, totalling 19 grams.

Thus, RM was certified by the Community Bureau of Reference (BCR) for its accepted reference value of cfp and testing for its homogeneity and stability. Stability was tested for normal storage temperature as well as higher temperatures to emulate transport conditions. To determine the accepted reference value, 12 laboratories were used to determine cfp on two different agar media: mannitol egg- yolk polymyxin agar (MEYP) and PEMBA. Based on results received from the testing laboratories, the assigned value was calculated, and RM was declared as CRM. The reconstitution of capsules also played a significant role in

recovery. There were various methods used for the reconstitution, like the placement of capsules in prewarmed PS solution, vortexing at various time durations, and analyzing the sample within 2 hours of reconstitution. The finalization of the reconstitution method for the prepared reference material is also crucial to have maximum recovery.

2.3 Microbial Preservation Techniques

Preservation of microbes is an essential step in the development of microbiological RM. The most common preservation method includes using a cold temperature (2 to 8°C) to store culture slants or plates with colonies followed by lyophilization (28). The main aim of all the preservation techniques is to slow down the metabolism of the microorganisms and use protectants to prevent further damage caused by the preservation techniques. Other preservation techniques are encapsulation, hydrogels, and hydrophilic or hydrophobic matrix creation for cell immobilization (29–31). New technologies like the cell alive system (CAS) technique, gelatin disk for transportation, microencapsulation by electrospinning, and electrospraying for intact microbiome preservation are helpful for the preservation of human gut microbes (32).

2.4 Protective Agents in RM

To develop reference material, one must produce it in a shelf-stable state. Since most of the microorganisms used in RM are prokaryotes, one must also be mindful of the stress triggered due to various preservation techniques like lyophilization, cryopreservation, etc. Several known protective agents are being studied and used to preserve the viability of the microorganisms. The well-known ones include dimethyl sulfoxide, polyvinylpyrrolidone (PVP), alcohols like glycerol, methanol, and sorbitol, protein-rich sources like blood serum, serum albumin, skimmed milk, peptone, yeast extract, and carbohydrates or their sources like saccharose, glucose, and malt extract (33). Gelatin has been used for preservation and storage of microbes like *Enterobacteriaceae*, *Neisseria, Streptococcus*, *Branhamella*, *Haemophilus, Gemella, Pseudomonas, Flavobacterium*, and *Bacteroides* species in the form of gelatin disks for a period ranging from 1 to 5 years at -20°C of

storage temperature (34). Essential factors in cryopreservation, particularly for pure culture, include the stage of cells and their physiological properties, which are dependent on their morphology, freezing and thawing rate, and protecting aid composition and concentration (35).

2.4.1 Cryoprotectants

Storage of microbial cells at freezing or ultra-low temperature is the most common type of microbial preservation, which results in the slowing of metabolic activity, leading to the survival of cells for a longer time. The general mode of functioning of cryoprotectant depends on its permeability. Macromolecules such as bovine serum albumin and SMP adsorb on the cell membrane, whereas small molecules such glycerol and dimethyl sulphoxide (DMSO) penetrate the cell membrane to offer protection from the outside environment changes (36). Most damage to the cells is brought by the unevenness of temperature distribution creating ice crystals which can have a detrimental effect on the microbial cells resulting in the loss of viability. Glycerol and DMSO are proven and commercially available cryoprotectants (32,33). Glycerol saline transport medium is used to preserve *Salmonella* and *E. coli* in fecal specimens during transport. At 4°C the survival is up to 4 weeks whereas for many years at -80°C (37). *E.coli* and *S. enterica* can be preserved with glycerol at -20°C for short-term preservation (1-3 years) and at -80°C for long-term preservation (up to 10 years). The lower the storage temperature, the longer the bacteria remain viable (38). Based on the experimental usage of cryoprotective agents, DMSO, methanol, ethylene glycol, propylene glycol and serum or serum albumin are the most effective cryoprotectants (39). Cryoprotective agents are generally species-specific, though glycerol-based cryoprotectants, along with nutritive agents, can act as universal cryoprotectants for single species and artificially created microbial groups (40).

2.4.2 Protein, Amino Acids, and Sugars

Different microorganisms have different needs, and some studies indicate that proteins, amino acids, and sugars play the role of protectants during cryopreservation or stress

conditions. Peptones and peptides are common names used for protein hydrolysates (41). Protein hydrolysates provide essential nutrients, including nitrogen, vitamins, and growth factors, which are vital for the cultivation of microorganisms. They are used in culture media to support the growth and maintenance of microbial cultures (42). The preferred sources of protein hydrolysates are by-products and waste produced after processing plants and animal products. The types of peptones and peptides present in protein hydrolysates depend on the biological source and manufacturing process. The protein hydrolysate manufacturing process involves hydrolysis using acids, alkalis, heat, enzymes, or microbial fermentation. Hydrolysis generates different types of bioactive peptides with antimicrobial properties (41,43,44). The most expensive ingredient in microbiological culture media is nitrogen, and studies have been conducted to evaluate FPHs produced from fish or shrimp waste and peptones from casein (casein hydrolysate), yeast, soy (soy protein hydrolysate), and gelatin as cost-effective alternatives (45–50). Catfish protein hydrolysate has been used in the formulation of microbiological secondary reference materials by food-testing laboratories under ISO 17025:2017 to maintain data accuracy and prevent false-negative reporting. Study involving 14% of fish protein hydrolysate (FPH) produced from catfish showed a better viability effect on *Salmonella* than milk protein (51). The literature available on the development of quantitative RM is scanty (18), and only one research article on the usage of protein hydrolysate in development of RM. The effect of three types of sugars and five types of proteins was studied by Dianawati et al. (2013) for microencapsulated *Bifidobacterium longum* 1941, which was subjected to freezing conditions (52). Based on this study, it was concluded that 12% whey protein concentrate, 12% Sodium caseinate, along 3% w/v glycerol were most effective in maintaining the viability of *B. longum* 1941 up to 99.2% during freezing conditions of storage. Hence, a combination of different proteins and sugars can have a better effect on the viability and act as a better protectant. There has been substantial research work conducted to identify protective agents and their optimal concentration for *Lactobacillus* cultures, which are primarily used to improve gut health. Protective properties of galacto-oligosaccharides have been described in preserving *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333

(29). The composition of lactose, galactose, and glucose in the commercially available galacto-oligosaccharides was necessary for the protective nature. The presence of a higher percentage of lactose, galactose, and glucose along with galacto-oligosaccharides resulted in higher protection of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333.

Glucose may not act as a primary cryoprotectant, but it can play a supportive role in the preservation of *E. coli* and *Salmonella* at low temperatures. Glucose (1%) when used in combination with cryoprotectants like glycerol can enhance bacterial survival rate during storage at freezing temperature (53,54). Trehalose is a disaccharide known for its protective properties, making it useful in the preservation of bacterial cultures. Trehalose maintains osmotic balance within the cells, which is crucial during the dehydration and rehydration processes associated with freezing and thawing. The role of trehalose in the growth and survival of *S. enterica* at high temperatures has been studied. The study found that trehalose accumulation is crucial for maintaining cell viability under stress conditions, including freezing (38,55). The addition of trehalose with glucose resulted in minimal disruption of protein expression, which resulted in effective cryopreservation of yeast (56).

2.4.3 Buffers

Various researchers have listed phosphate buffer and peptone saline as effective media for maintaining the viability of microbial cells. Peptone water (0.1%), phosphate buffer saline, maximum recovery diluent, etc. have been widely studied and demonstrated to be effective for the removal of toxic metabolic wastes or byproducts released by microbial cells, which in turn negatively affect cell survival (30,57,58). The revival or reconstitution of preserved cultures is an essential step in RM development and applies to various proficiency testing schemes. RM manufacturers in microbiology also provide instruction methods for reconstitution, which lists the procedure containing a type of recovery diluents and microbiological media for better recovery. The instruction also mentions the reconstitution period and time limit within which the reconstituted RM needs to be used or cultured. Nutritive microbiological media like tryptic soya broth/agar have also been tested to be good media for recovering microorganisms. Tryptic soya broth (TSB) is effective for the

reconstitution stage, whereas tryptic soya agar (TSA) has been found to give better recovery at the colony formation stage (59).

2.5 Development of Formulation for Quantitative RM

The primary objective in formulating quantitative RM is to achieve a fixed value of microbial cells that remain viable and culturable over a significant period. Preservation techniques, such as cold temperature storage and encapsulation, are effective tools for controlling microbial cell growth. These techniques are complemented by the inclusion of protective agents to prevent cell damage, as noted by various researchers in past (52,60). The main key to the development of formulation for quantitative RM involves selection of nutritive and protective agents for long-term survival of the target micro-organism along with maintaining homogeneity and stability. Based on the available scientific literature on preservation of cultures, the three key factors were selected for inclusion in formulation, nutritive agent, and protective agent to maintain cell membrane stability to avoid cell death and ease in resuscitation, and preservation technique utilizing lower temperature and encapsulation to maintain cell dormancy. Figure 2 below gives a schematic representation of the different components used for the development of the quantitative RM in this study.

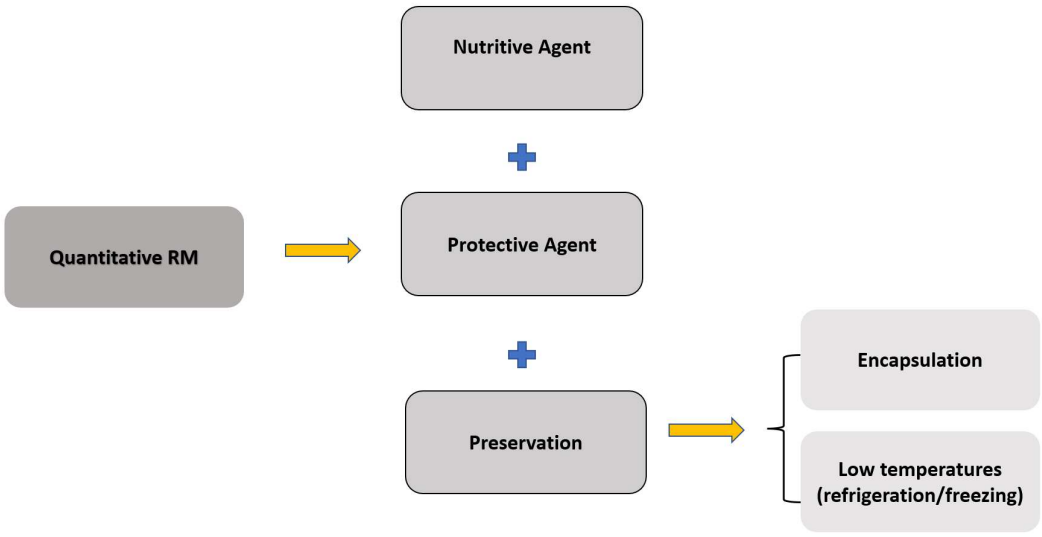


Figure 2. Schematic representation of components used in development of quantitative reference material formulation.

2.6 Utilization of Response Surface Methodology

Response Surface Methodology (RSM) has been used in optimization study that involves more than two variables for process and product optimization using designed experiments (61). The selection of RSM was based on the opportunity to significantly reduce experimental runs without compromising the study, as it establishes a correlation between the different variable factors and extrapolates to provide the relationship (if present) between the different factors in the study (62). The RSM software uses a quadratic statistical model and utilizes Analysis of Variance (ANOVA) for the assessment and verification of the proposed model by the software. For the design of the experiment, the Box-Behnken Design (BBD) was used, as the experiment involved more than three factors (63). The statistical model codes the variable factors and the selected concentration in a three-level factorial design, which is -1, 0, +1, where 0 represents the center point (64).

2.7 Determination of Homogeneity, Stability and Assigned Value of RM

2.7.1 Determination of Homogeneity

For any format of RM, traceability to the metrological SI unit is mandatory. This implies that the microbial strain and the equipment used in the characterization establish an unbroken chain of traceability. Estimation of uncertainty measurement is required to determine the certified or assigned value of the RM. For estimation of assigned or certified values in microbiology, Log base 10 values are used for CFU counts. The precision value of CFU obtained in microbiology studies is very extensive as it involves viable cells that are widely distributed or non-homogeneous in food matrices. As per ISO standard, assessment of homogeneity and stability is crucial for RM samples and is a bit tedious when it comes to microbiology RM (5). For estimation of homogeneity, sampling is done from the pool of RM prepared. Guidance on the number of samples to be drawn can be taken from ISO 33405:2024 for homogeneity and stability assessment (65). Units or samples selected from the produced RM batch for homogeneity assessment should have an appropriate statistical representation of the population or batch of produced RM. As per ISO 17034:2016, the units can be chosen, for example, by random selection, stratified

random selection, or systematic selection from a random start point (5). If different batches are produced, then the sample needs to represent all the produced batches. Different microbiological media and diluents need to be used during this homogeneity assessment as the recovery depends on the nutritional value of the media used. TSA or nutrient agar and diluents like TSB are used for enrichment steps that can increase the recovery from RM samples. Different statistical tools are used for homogeneity assessment, like an estimation of the standard deviation in the observed reading. As per ISO 13528:2022, the CFU value obtained should follow a binomial distribution or be within the Poisson distribution (66). Other values like mean values and fractions of negative value or % negative, where no recovery or values are different from the expected value, also need to be calculated for homogeneity assessment.

Homogeneity formula $$T_{\chi} = \Sigma \frac{(Yi - Y+/I)^2}{(Y+/I)} \qquad (67)$$

Where Yi is the count observed for one capsule and Y+ is the total count from all capsules. The T_{χ} is homogeneity and will follow the X^2 distribution with I-1 degrees of freedom (I = number of samples tested).

Different laboratories can also be used as part of a collaborative study of reference material, which also helps to understand the performance of RM in different conditions and different testing methods, and arrive at an accepted reference value for the developed RM (9).

2.7.2 Determination of Stability

Stable RM in microbiology is challenging, as we know the impact of temperature and time on viable cells. A shelf-life study needs to be conducted to assess the stability of microbiology RM. ISO provides details on stability determination. The evaluation of data obtained during the stability study can be analyzed for linear regression and the analysis of variance (66). A two-tailed t-test can be used to determine a significant difference in the value of replicate testing (57). The calculation of recovery is not listed in the ISO

17034:2016 document, but it has been calculated in some research papers where stability is monitored over a long period (5). Recovery (%) can be calculated based on the initial inoculum value and counts observed at different time intervals during storage (58,68).

2.7.3 Calculation of Assigned Value

To calculate the assigned or certified value, expert laboratories can be given RM samples to determine the assigned value (69). ISO 13528:2022 elaborates on the determination of assigned value and standard uncertainty by different approaches, like knowing the amount or value used for spiking or formulation, applying certified reference value, the result obtained from one laboratory, and a consensus value from expert laboratories (66). The limitation of each approach determines the selection of a suitable approach for calculating the assigned value. Formulation and certified reference value approach can only be applied if the analyte is homogeneously distributed in the food matrix and the availability of certified reference material of the target analyte (66). Estimation of the uncertainty budget is a necessary calculation that is included in the assigned or certified value and ISO 19036:2019 can be referred for this purpose in microbiology (70). To establish the value for a certified reference material (CRM), it is essential to use measuring equipment, glassware, pipettes, etc., that are traceable to the International System of Units (SI). These instruments must have an unbroken chain of traceability leading up to a National Metrological Institute (NMI). Additionally, when using a culture strain, it must be traceable to a recognized culture collection.

2.8 Current scenario & limitations in PT sample availability

A brief overview of different microbiological Proficiency Testing Providers (PTPs) accredited by accreditation bodies for ISO 17043:2023 has been provided in Table 3. Several PTPs provide PT samples of different food matrices with different microorganisms. One of the popular formats of food matrix is lyophilized or freeze-dried powders used for creating PT samples. The probable reason for selecting this type of format is the ease of attaining homogeneity and stability of PT samples as they contain

microorganism(s) in the food matrix. Even in a single source water sample with indicator microorganisms, there can be variations seen due to random causes (71). Unexpected variations, or random causes, can often be attributed to the presence of living microorganisms, which result in a non-homogeneous sample. For proficiency testing (PT) schemes involving drinking or potable water, proficiency testing providers (PTPs) typically use pure cultures in a lyophilized state. Participants then add water to these cultures before analysis, which facilitates the ease of transporting water PT samples. For spices, the American Spice Trade Association (ASTA) provides a PT sample for *Salmonella* spp. in black pepper, which is in powder form. A similar format is provided by other PTPs, representing the spice matrix. From the data available on different food matrices used by PTPs to create PT samples, it can be concluded that a representative food matrix is used to represent a food segment. For example, lyophilized meat powder and lyophilized egg powder are used to represent meat and meat products and egg and egg products, respectively. Extended use of PT samples for capability development or troubleshooting in developing countries of Asia Pacific (APAC) is limited due to its high cost since most of the PTPs are situated in the USA and the EU. Hence, there is a need for an increase in the variety of PT samples with different food matrices, focusing on increasing the numbers of PTPs in the APAC region.

Table 3*:* Overview of Proficiency Testing schemes offered by accredited Proficiency Testing Providers across the globe

Country	Accreditation Agency	Proficiency Testing Provider	Matrix (Food/Water/Environment)	Source
USA	A2LA	Advanced Analytical Solutions, LLC	Drinking Water, Non-potable water & environmental samples	https://customer.a2la.org/index.cfm?event=directory.index
USA	A2LA	AIHA Proficiency Analytical Testing Programs	Environmental (pure culture)	
USA	A2LA	AOAC International	Mashed potatoes, ground and processed meat	
USA	A2LA	Cherney Microbiological Services, Ltd. A wholly-owned subsidiary of Matrix Sciences International, Inc.	Rehydrated mashed potato and whey powder	
USA	A2LA	ERA	Drinking Water, Non-potable water & environmental samples	
USA	A2LA	New York State Department of Health Wadsworth Center	Drinking Water, Non-potable water & environmental samples	

USA	A2LA	Phenova, Inc.	Drinking Water, Non-potable water, solid & chemical materials & environmental samples	
USA	ANAB	Matrix Sciences International Inc	Pure culture (no matrix involved)	https://search.anab.org/
USA	ANAB	Moffett Proficiency Testing Laboratory, Division of Food Processing Science & Technology, Office of Food Safety, Center for Food Safety	Raw milk, finished milk products, Shellfish Growing Area Waters, food products (no detail on matrix type provided in scope), and animal feed.	
USA	ANAB	NSI Lab Solutions, Inc.	Cannabis, Cannabis Products, and Edibles, Food Items (no detail on matrix type provided in scope), drinking water, non-potable water & environmental samples	
USA	ANAB	Sigma Aldrich RTC, Inc.	Drinking water, non-potable water, environmental samples, Simulated Drinking Water, wastewater, Food & Beverage (no detail on matrix type provided in scope)	

USA	ANAB	The University of Arizona, Aquaculture Pathology Laboratory	Crustaceans	
Canada	A2LA	Clinical Microbiology Proficiency Testing	Drinking Water & recreational water	https://customer.a2la.org/index.cfm?event=directory.index
Canada	A2LA	Proficiency Testing Canada Inc.	Water	
Germany	A2LA	Deutsches Referenzburo fur Ringversuche und Referenzmaterialien GmbH (DRRR GmbH)	Milk, cheese, milk powder, fruit preparation, animal feed, food contact material, re-cooling water,	
Belgium	economie	ILVO – EV (Eigen Vermogen VH Instituut Voor Landbouw- EN visserijonderzoek)	Raw milk	https://economie.fgov.be/en/themes/quality-and-safety/accreditation-belac/accredited-bodies/proficiency-testing-organizers
		Prins Leopold Instituut Voor Tropische Geneeskunde	animal tissue (pigs)	
		Réseau Qualité SUD (REQUASUD) ASBL	Smoked salmon, Ham sausage, UHT milk, Soy milk	

Germany	DAkks	Robert Koch-Institut ZBS	Food (no details on matrix type provided in scope) & environmental samples	https://www.dakks.de/en/accredited-bodies-search.html
UK	UKAS	Fera Science Limited (FAPAS)	Meat, Chicken, Rice, Flour, Salad, Fruit juice, Fruit, Egg, Fish, Milk & Milk powder, Infant formula, Pepper, Confectionery (including chocolate), Cocoa powder, Cheese, Ready to eat meal, Herbs and spices, Mixed vegetables, Animal feed, Environmental and sponge swabs, Potable water, Recreational water	https://www.ukas.com/find-an-organisation/browse-by-category/?cat=368
UK	UKAS	LGC Limited	Lyophilized test material, Food products (milk powder, oatmeal, tea, herbs, spices, etc.), skimmed milk powder, meat, and fish products, dairy and meat products, chocolate products, gelatine hydrolysate, animal feeds – Oatmeal, simulated water, and environmental products, plastic surface and plates, alcoholic beverages (beer), beverages – non-alcoholic, sugar.	
		UK Health Security Agency, Food and Environment Proficiency Testing Unit	Freeze-dried format and control dried (Lenticule®) – pure culture	

France	cofrac	ASA - Animal Societe Aliment	Milk powder, gelled food matrix	https://tools.cofrac.fr/en/easysearch/resultats_advanced.php
France	cofrac	BIPEA - Bureau Interprofessionnel D'etudes Analytiques	Meat products (no detail on matrix type provided in scope), alcoholic beverages, fishery products (no detail on matrix type provided in scope), soft drinks, fresh or frozen fruit	
Australia	A2LA	IFM Quality Services Pty Ltd.	Ready-to-test, dry food materials. Content varies with each round, e.g., soup mix, custard powder, infant formula, breadcrumbs, breakfast cereals, etc. Ready-to-test skim milk powder, chocolate pieces, Fresh meat patty (freeze-dried), Fresh seafood paste (freeze-dried), Ready-to-test herbal preparation, freeze-dried vial of culture, swabs	https://customer.a2la.org/index.cfm?event=directory.index
Vietnam	A2LA	National Institute for Food Control	Water (Bottled, mineral water, and domestic water), beverages (Soft drinks, alcoholic beverages), milk, meat & meat products, fish and fish products (no detail on matrix type provided in scope)	
Vietnam	A2LA	Quality Assurance and Testing Center 3 (QUATEST 3)	Food and feedstuff (Meat, milk, cereal, aquatic products, nutritious powder, beverages, feedstuff, etc.) and water	

Vietnam	A2LA	Quality Assurance of Vietnam	Milk Powder, Milk Product, Animal Product, Fishery Product (no detail on matrix type provided in scope)	
Japan	PJLA	Japan Food Inspection Corporation Tokyo Metoropolitan Area Office	Quantitative (no details provided on matrix type in scope)	https://www.pjlabs.com/search-accredited-organizations
China	CNAS	Guangzhou Customs District Technology Center	Food simulants（lyophilized powder）	https://las.cnas.org.cn/LAS_FQ/publish/externalQueryPTEn.jsp
China	CNAS	Technical Center of Shenyang Customs	Freeze-dried powder/artificial food	
China	CNAS	Analysis Capability Assessment System of Chinese Academy of Inspection & Quarantine	Milk powder	
China	CNAS	National Institutes for Food & Drug Control	Freeze-dried powder (simulated food), Freeze-dried fungus ball	
China	CNAS	Technical Center of Qingdao Customs District	Simulated foods (Freeze-dried powder)	

China	CNAS	Science and Technology Research Center of China Customs	Lyophilized powder (Simulated food)
China	CNAS	CFAPA Testing Technology Company Limited of Dalian	Simulated food, milk, and milk products (no detail on matrix type provided in scope)
China	CNAS	Shanghai Institute for Food and Drug Control	Food (no detail on matrix type provided in scope)
China	CNAS	Technology Centre of Dalian Customs District	Food and Fodder (no detail on matrix type provided in scope)

2.9 Survival of Pathogenic *E. coli* & *Salmonella* in Food Matrices

In simple terms, a microbiological PT sample should have a target analyte in homogeneous and stable form without any drastic change in its numbers and unaffected by the food matrix in which it is present. Since this food matrix is not the ideal environment for the growth of the target microorganism, survival becomes important. Also, the food matrix should not be conducive enough to allow growth of the target analyte as such conditions limit quantitative PT study. Microorganisms have evolved and developed adaptability while growing in extreme conditions and environments. While searching for articles on the development of microbiological PT samples in scientific literature, the search results are limited. There were only 402 articles available in the Scopus database with 'proficiency testing' as a keyword search in the subject area of 'Microbiology and Immunology'. This constitutes only 0.06% of all articles available in the Scopus database with 'proficiency testing' in the keyword search. Moreover, such studies, including homogeneity and stability studies, are trade secrets of PT manufacturers. So, to provide useful information that can be used to create and develop PT samples, information and data were sourced from food safety incidents, food recalls, and survival studies of pathogens in food matrices. Troubleshooting food safety incidents also provides insightful information on favorable factors of food matrices, which can be extrapolated and used to design development studies for microbiological PT samples. To understand food pathogen survival better, pathogens, food matrices, and their interactions were studied. Hence, the factors that can aid in the survival of food pathogens and the effect of food matrices that can positively impact the survival of *Salmonella* spp. and *E. coli* were categorized and discussed in the following section. These factors are selected by their degree of influence on the survival of *E. coli* and *Salmonella.* During the study of these favorable factors, it was noted that some of the factors are interdependent and hence clubbed together. Factors like time, temperature, and relative humidity are interdependent, as put forward in various survival studies of pathogens in food matrices. Some of the intrinsic factors of food matrices like nutrient content, antimicrobials, and moisture level of food may or may not be interdependent. We have also tried to correlate the selected factors with the common practices observed to be

followed by PTPs viz., storage of PT samples at low temperature, avoiding the presence of competitor microorganisms in one PT sample, and preference of low water activity food matrices like lyophilized food or a freeze-dried powder to attain better homogeneity and stability (15). Figure 3 summarizes the factors that need to be considered during the development of PT samples for better survival of food pathogens. These factors, which influence the development of PT samples, are inoculum (level, strain type, growth phase, and preparation), storage conditions (temperature, time, pH, and relative humidity), protectants, and the kind of food matrices used. The details of the impact of these factors on the survival of *E. coli* and *Salmonella* are discussed in the sections below.

2.9.1 Effect of inoculum level, strain type, growth phase & inoculum preparation on survival

The survival of any micro-organism is greatly influenced by the initial quantity or level at which the said micro-organism is added to a food matrix. The survival rate of *E. coli* O157:H7 and *Salmonella* spp. inoculated on freshly hulled in-shell walnuts at 8 Log CFU per gram of each pathogen and stored for 12 months at 10°C and 65% relative humidity (RH) was studied (72). During the initial 8 days of storage, *E. coli* O157:H7 was detected at 2.86 Log CFU per gram, while *Salmonella* spp. exhibited a count of 4.4 Log CFU per gram. After 360 days, *Salmonella* spp. showed only 0.79 Log CFU per nut, whereas some samples fell below the limit of detection for *E. coli* O157:H7. This suggests that *Salmonella* spp. may have better survival under the given conditions compared to *E. coli* O157:H7. A similar study on the impact of inoculum levels of *Salmonella* spp. and *E. coli* O157:H7 was reported in different nut matrices (in-shell walnuts, almonds, pistachios, pecan nutmeats, and pecan kernels), where low inoculum levels led to a significant decline in the survival rate of both pathogens. A better recovery rate is expected if the initial inoculum level is high, i.e., 7 – 11 Log CFU per gram, combined with storage at a lower temperature of 4°C as compared to a higher temperature of 23°C (73–77). Different *Salmonella* strains show different survival levels in different food matrices; for example, *S.* Oranienburg did not show survival on almonds and pistachios post 72 hours of inoculation drying and

storage at -19°C or 24°C with 7.3 – 7.5 Log of initial inoculum as compared to *S.*Anatum, *S.* Enteritidis PT 9c, *S.* Enteritidis PT 30, *S.* Montevideo, *S.* Tennessee. The raw almond kernels were inoculated with *S.* Enteritidis Phage Type 30 (8 Log CFU per almond) and stored at -20°C and 4°C for 550 days, showing no significant reduction in the counts, thereafter it can be concluded that for some species of *Salmonella*, larger inoculum value is required for survival in nuts (74).

The initial steps or conditions introduced during inoculum preparation also affect the survival of microorganisms significantly under unfavorable conditions of different food matrices. In a study, researchers observed that the inoculum from agar plates exhibited greater resistance to drying compared to the inoculum taken from broth. This finding suggests that using inoculum from agar plates may lead to a better survival rate during inoculation (77). Lyophilized culture of *S.* Anatum, originally isolated from milk chocolate by the FDA, was found to have better heat-resistant capacity, supporting the prevention of initial osmotic shock of culture when inoculated in milk chocolate (78) Conventional testing of a foodborne pathogen from food samples involves selective enrichment steps, resulting in a preference for environmental strains rather than pathogenic human-origin strains (79). During the initial inoculum growth of *E. coli* O157:H7 and *S.* Typhimurium on restrictive nutrient media (M9), researchers observed an increased adhesion of both pathogens to the spinach surface (80), thus increasing their ability to survive.

Starvation during the stationary phase and alkaline pH induces *E. coli* to express the production of phage shock protein, which can also be induced by other environmental stress conditions, viz., heat shock, osmotic shock, ethanol treatment, and filamentous phage infection. These phage shock proteins have been shown to increase the survival of *E. coli* in nutrient-deprived conditions (81). Similarly, cold shock before freezing *S.* Enteritidis at 10°C resulted in its better survival rate (82). Under environmental stress of low temperatures, *E. coli* and *S. enterica* serovar Enteriditis adapt to filamentous phenotypes due to gene mutation for cell division (83–85). Filamentation has also been observed for *S. enterica* serovar Oranienburg in response to low water activity (83), whereas *S. enterica* serovar Paratyphi showed filamentous phenotype due to stress included from the

presence of salts in solid media (86). The cells in their exponential stage have shown greater injury levels due to sub-lethal stress than stationary cells as sub-lethal stress produces a burst of intracellular free radicals, which are responsible for the injury and death of microbes (87).

Based on the survival studies data, different strains of *Salmonella* show different survival rates when subjected to the same food matrices. Hence, for manufacturing PT samples, a PT provider needs to have a complete understanding of the survival rate and performance of the selected strain. If the survival rate is not as expected, PTPs can change the strain to a more robust surviving strain, such as an environmental strain rather than human pathogenic strain, in the design of the experiment. Similarly, for better survival, the inoculum of *E. coli* & *Salmonella* can be subjected to stress by cold or heat shock, nutrient deprivation, selection of lag phase culture, and a higher inoculum of 7 – 11 log/gram can be considered for better survival in the PT sample.

2.9.2 Effect of storage temperature, time, relative humidity & pH on survival

Various studies have shown that environmental conditions have a great impact on the survival of *E. coli* and *Salmonella*. A study investigated the effect of storage temperature on *Salmonella* spp. inoculated in walnuts (73). Results indicated that a storage temperature of 4°C was more effective for pathogen survival compared to storage at 23°C. Additionally, a low initial inoculum of less than 400 CFU per nut was recovered after 3 months of storage. Long-term survival of *Salmonella* spp. in tree nuts has also been well studied and documented (74,77,88). *Salmonella* spp. can survive better at cold temperatures, and in some cases, it was observed that no changes in cell count occurred after one year of storage at − 20°C or 4°C (74,75,77,88). Studies conducted with an increase in temperature from − 20 to 5°C result in the decline of *Salmonella* spp. in pecan kernels, crushed cocoa shells, and hazelnut shells (75,89,90). *Salmonella* spp. can survive for a long period under reduced water activity supported by lower temperatures (91). The survival rate of foodborne pathogens is negatively impacted by an increase in storage temperature and time. The most

common storage temperature range that PTPs follow for microbiological PT samples is from -20°C to 4°C, with a maximum storage time of six months to one year.

Relative humidity during storage can play an essential role as higher humidity of 85 – 95% can influence the moisture level of the food matrices due to the equilibrium phenomenon, resulting in increased moisture content of the food matrices (92). With controlled temperature maintained at the PT provider end, the ideal relative humidity for PT samples is between 30 - 65% during the storage period.

Apart from temperature, the pH of food matrices also significantly impacts the survival of foodborne pathogens. Survival of foodborne pathogens, including *S.* Typhimurium and *E. coli* O157:H7, was studied in different strength beer, where they were only able to grow in alcohol-free beer, whereas in mid-strength beer, pathogens could survive for 30 days at 4°C. Lower temperature favored the survival of pathogens in all types of beer, but a pH drop from 4.3 to 4.0 in alcohol-free beer prevented any growth of the pathogens (93). In another study, stationary phase cells of non-pathogenic strains of *E. coli*, *E. coli* O157:H7, and *Salmonella* spp. were subjected to acid adaptation to pH 5 in Tryptone Soy Broth (TSB) at 37°C for 4 hours and then inoculated in ketchup, mustard, and sweet pickle relish which are acidic food matrices. Acid adaptation enhanced the survival of *E. coli*, including pathogenic strains of *E. coli* O157:H7, and *Salmonella* spp. at 5°C in ketchup (Tsai & Ingham 1997). *E. coli* O157:H7 shows an enhanced ability to survive in acidic conditions of pH ≥4.0, and survival is positively influenced by the presence of specific acidulants, which include hydrochloric acid, acetic acid, lactic acid, and also by lower temperature, and the type of strain (94,95). Lower pH is generally considered as detrimental to the survival of food pathogens, hence for making PT samples with acidic food matrices, acid adaptation, and low temperature can be considered for aiding better survival of pathogenic strains of *E. coli* & *Salmonella* in low pH food matrices.

2.9.3 Influence of protectants on survival

The presence of glucose-fructose in growth media with 0.95 water activity for 12 hours can develop heat resistance in *Salmonella* spp. (91). Similarly, the positive influence of sodium

chloride or glycerol can be observed in the development of heat tolerance in *Salmonella* spp. (91). The significance of the broth base for the survival of *Salmonella* spp. was highlighted by a study. It was found that 8% NaCl in Nutrient Broth exhibits bactericidal effects against *Salmonella* spp., whereas the same concentration of NaCl in TSB (Tryptic Soy Broth) did not have bactericidal properties (91). Similar results were demonstrated where an increase in NaCl concentration in broth, representing food dressing or sauce containing acidic food, increased the survival of *E. coli* O157 SERL 2 (96). In a similar study involving different strains of non-pathogenic *E. coli* and *S. enterica*, researchers found that a 0.5% concentration of NaCl and 10% sucrose exhibited a protective effect against the lethal concentration of acetic acid for all strains of *Salmonella* and *E. coli*. (97). Protonated forms of acetic acid, malic acid, and L-Lactic acid have been shown to have a protective effect on *E. coli* O157:H7, where D-Lactic acid has the most protective effect at 1 to 20 mM concentrations (98). Protective solutes can be selected and considered for inclusion in PT samples to increase the survival of *E. coli* & *Salmonella* in food matrices.

2.9.4 Significance of water activity on survival

Low moisture or low water activity food is generally categorized as food having a water activity of <0.6. To include more varieties of low moisture food matrices (nuts, marshmallows, jerky, nougat, mint, and dried sausages), low water activity food can also be assigned within the range of 0.6 to 0.85 (99,100). For optimal functioning and growth, microorganisms require water activity of 0.990 to 0.995. The water activity of 0.983 is inhibitory for *Salmonella* spp. whereas under 0.95 has been reported to be detrimental to the growth of *E. coli*. However, when solutes (NaCl) were used for adjustments, the minimum water activity for optimal growth of *Salmonella* spp. and *E. coli* was 0.950 (101–103). Foodborne pathogens can remain viable and survive for a prolonged period in dry foods, raw materials, and food contact surfaces in food manufacturing environments, for example, steel and plastic surfaces, equipment, and food handling personnel (104–106). *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) have been shown to survive when dried on paper discs for 35 days at 35°C, whereas their survival rate increased

to 70 days at 25°C and was extended to 24 months at 4°C, suggesting prolonged survival at lower temperatures (107). Another study of the transfer of *S. enterica* via artificially contaminated salt of 0.49 water activity showed survival for 146 days (108). *E. coli* O121 and *Salmonella* cocktails in wheat flour showed survival from ~7 logs on day one to ~2 logs on the 360th day (109). One of the biggest challenges bacteria face in low water activity foods or dry conditions is managing water content within their cell and avoiding as much as possible damage to their cell components, such as DNA and protein components, including structures, i.e., the cell membrane, which is essential for their viability. The survival process for bacterial cells involves tackling three different stages, which include initial acclimatization to dry conditions, dehydration, and avoiding sudden changes in environmental conditions during resuscitation. A maximum number of cell deaths occur during the initial drying process as the bacterial cells need to react immediately to loss of turgor and cell membrane damage. With worsening drying conditions, bacterial cells maintain basic survivability by resisting oxidative damage and avoiding DNA damage. The presence of specific solutes, i.e., sucrose and trehalose in the medium during drying or low moisture conditions has shown a positive influence on the survivability of bacterial cells (107,110). The positive influence of these compatible solutes can be attributed to the increased accumulation in bacterial cells because of their low molecular weight. This process is supported by limiting further water loss without limiting the cellular process (111,112).

Predictive modeling systems designed to assess the survivability of different foodborne pathogens in various food matrices have consistently identified pH and water activity as key influencing factors (113). Certain strains of STEC and *E. coli* O157:H7 are resistant to acids that can enhance survival in pepperoni, which is a low-moisture food (114). Though the accurate mechanism of survival of *Salmonella* spp. under low water activity conditions is not fully known, it can be attributed to lipopolysaccharide (LPS) present in the outer cell membranes (115). Higher water activity results in a higher death rate of *E. coli* O157:H7 and *Salmonella* spp. (116). In various studies, *Salmonella* spp. has shown more excellent heat resistance in low water activity than in high water activity

(117,118). Similarly, *Salmonella* spp. can survive in low moisture food with high-fat content and high sugar products (119–121).

2.9.5 Impact of food matrices on survival

Food matrices are categorized into food groups or food types based on the major ingredient(s) present in the composition of the food. The different components or ingredients present in the food can influence the growth and survival of microorganisms.

2.9.5.1 Influences of different ingredients of food matrices

S. Typhimurium survival rate was studied in regular and fat cheddar cheese, where the decline of *S.* Typhimurium was observed to be faster in fat-reduced cheese. The study further suggested that the reduction of fat levels from 48 to 36% resulted in a significant decline in the initial inoculum (122). A study of foodborne outbreaks involving low infective doses of *Salmonella* spp. highlighted the involvement of high fat and high protein food matrices, suggesting that such food matrices are protective against low levels of pathogens (123–125). Milk solids in skimmed milk can significantly increase heat resistance in *Salmonella* spp. and *E. coli* O104:H7. Lower percentages of milk solids (10%) were found to be effective in the survival of *E. coli* O104:H7, whereas higher percentages of milk solids (42 and 51%) were found effective for *S.* Typhimurium and *S.* Alachua (126). A study conducted to determine the reduction rate of 6 Log per gram of *S.* Eastbourne and *S.* Typhimurium in chocolate bars showed that *S.* Typhimurium was less resistant as compared to *S.* Eastbourne, as it could be recovered from chocolate bars after nine months of storage (127). Bitter chocolate showed more reduction in both strains as compared to milk chocolate. Similarly, meat meal was observed to provide better survival of *Salmonella* spp. as compared to fish meal and casein when inoculated with strains of *Salmonella*.

Microbial contamination of matrices in the laboratory to create a PT sample is not similar to natural contamination. In natural contamination, protection is provided to microbes since they are embedded within the protein and lipids of the food matrices (128). So, to avoid the loss of microbial cells, artificial contamination in the lab should mimic

natural contamination as far as possible. *S. enterica* serovars were observed to be resistant to heat treatment up to 90°C in peanut butter and showed only a 3.2 Log reduction of the added inoculum (129). The heat resistance of *S. enterica* serovars was attributed to the colloidal nature of peanut butter, of fat and water in the peanut meal phase. Three serovars of *Salmonella* (*S.* Typhimurium, *S.* Newport, and *S.* Montevideo) were inoculated in sesame seeds and tahini (paste of sesame seeds) with 5 – 6 Log CFU per gram. The complete reduction was seen in sesame seeds, whereas only 4.5 and 3 Log reductions were seen for tahini post 16 weeks of storage at 22°C and 4°C, respectively. Suggesting tahini as a better matrix for *Salmonella* serovar survival (130). Similarly, powdered infant formula was observed to be a better matrix, resulting in the survival of sub-lethally injured cells of *S.* Enteritidis and *E. coli* for 15 months (131). Shredded iceberg lettuce supports enhanced growth of *E. coli* O157:H7 and *S. enterica* serovars Typhimurium compared to uncut lettuce and water at 22°C (132). Cut surfaces can act as a harborage site for *E. coli* O157 and *Salmonella* spp. by providing a site for attachment and internalization (133).

For manufacturing PT samples, PTPs use a representative of a particular food segment or category such as to represent (1) scope of testing (12). Selection of a particular food matrix that can act as a representative of a particular food category or segment needs to be made cautiously to facilitate the survival of the pathogen.

2.9.5.2 Inhibitory effects of food matrices

Spices and herbs are food matrices that exhibit antimicrobial or bacteriostatic activity. Most processed food matrices have preservatives in their formula, which are used to increase the shelf life of the products. Peppercorns and fenugreek seeds have shown survival of spore-formers, coliforms, and pathogens such as *E. coli* and *Salmonella* spp. irrespective of the widely reported antimicrobial effect of spices and herbs (134). *E. coli* O157:H7 and *S.* Typhimurium have also been reported to have resistance to the antimicrobial effect of cranberry juice (135).

Clove and garlic have shown better bactericidal activities as compared to mustard and ginger, which are bacteriostatic for *E. coli* O157 and *S.* Enteritidis (136). Six different

strains of *Salmonella* and *E. coli* O157:H7 were inoculated on fresh culinary herbs and stored at 4°C for 24 days to study the survival rate. Cilantro showed better survival when compared to rosemary (137). For an antimicrobial compound to be effective against microbes, it needs to be metabolized by them. Hence, during the physiological dormant state of foodborne pathogens induced due to stress and starvation, there will be limited efficacy of antimicrobials, which in turn explains the survival of foodborne pathogens in herbs and spices that contain natural antimicrobials.

The adaptation study of *E. coli* has shown that it can adapt to a higher weak acid concentration of sodium benzoate and potassium sorbate in a stepwise manner of 1000 ppm with repetitive culturing can lead to adaptation to 7000 ppm (138). Mustard flour (10 to 20%) and acetic acid (0.5%) show retardation in the antimicrobial effect of mustard against *E. coli* O157:H7 (139). For facilitating the survival of pathogenic & non-pathogenic strains of *E. coli* & *Salmonella* in the presence of antimicrobials, stress-induced cultures, stationary or lag phase cultures, and lower-dose antimicrobial adaptation, along with protectants, can be considered.

2.9.5.3 Presence of Other Microorganisms

Certain pathogens are negatively influenced by the presence of other microorganisms. For example, *S.* Typhimurium is negatively influenced by the presence of *Streptococcus* and lactic acid bacteria due to the production of hydrogen peroxide, lactic acid, and bacteriocins produced by the latter (87). *S.* Enteritidis on chicken and *E. coli* O157:H7 on beef showed an increase in the growth on treated or decontaminated meat due to the absence of non-pathogenic competitor microorganisms, due to the treatment of meat (140). Similar studies of natural competitive flora in fresh river water and bank sediments showed that the survival of *E. coli* and *E. coli* O157:H7 is independent of competitive flora (141). However, the presence of stationary phase cells of *S.* Typhimurium and exponential phase cells of the same pathogen can develop resistance in exponential cells to stress and thus increase the survival rate of young cells. This phenomenon can be due to cell-to-cell interaction wherein old cells can impart resistance to young cells when present together in a single

medium (142). During the review of PT schemes provided by PTPs across the globe, it was noted that competitor microorganisms are avoided in the same PT sample. Most of the microbiological PT samples have single target microorganisms for qualitative and or quantitative determination. PTPs also offer schemes that can have more than two microorganisms belonging to the same group to minimize any effect from interactions between microorganisms from other groups. LGC Group offers schemes for *Enterobacteriaceae,* which include *E. coli*, Coliform (*Enterobacter*), and Total Mesophilic Count. Some schemes have Gram-positive microorganisms, viz., *Bacillus cereus* and *Staphylococci,* together in one PT sample. Based on the PT scheme information available on the websites of several PTPs, it can be concluded that the Gram nature of the microorganisms is considered for combining the microorganisms or target analyte in one scheme. Hence, Gram-positive and Gram-negative microorganisms are grouped separately in PT schemes by some of the PTPs. AOAC International provides separate PT samples for *Salmonella, E. coli* O157: H7, and *Listeria monocytogenes.* There are pathogen-free PT schemes provided by some of the PT providers, where *E. coli*, Coliform, *Enterobacteriaceae*, Aerobic Plate Count, Yeast and Mould are combined in one PT sample.

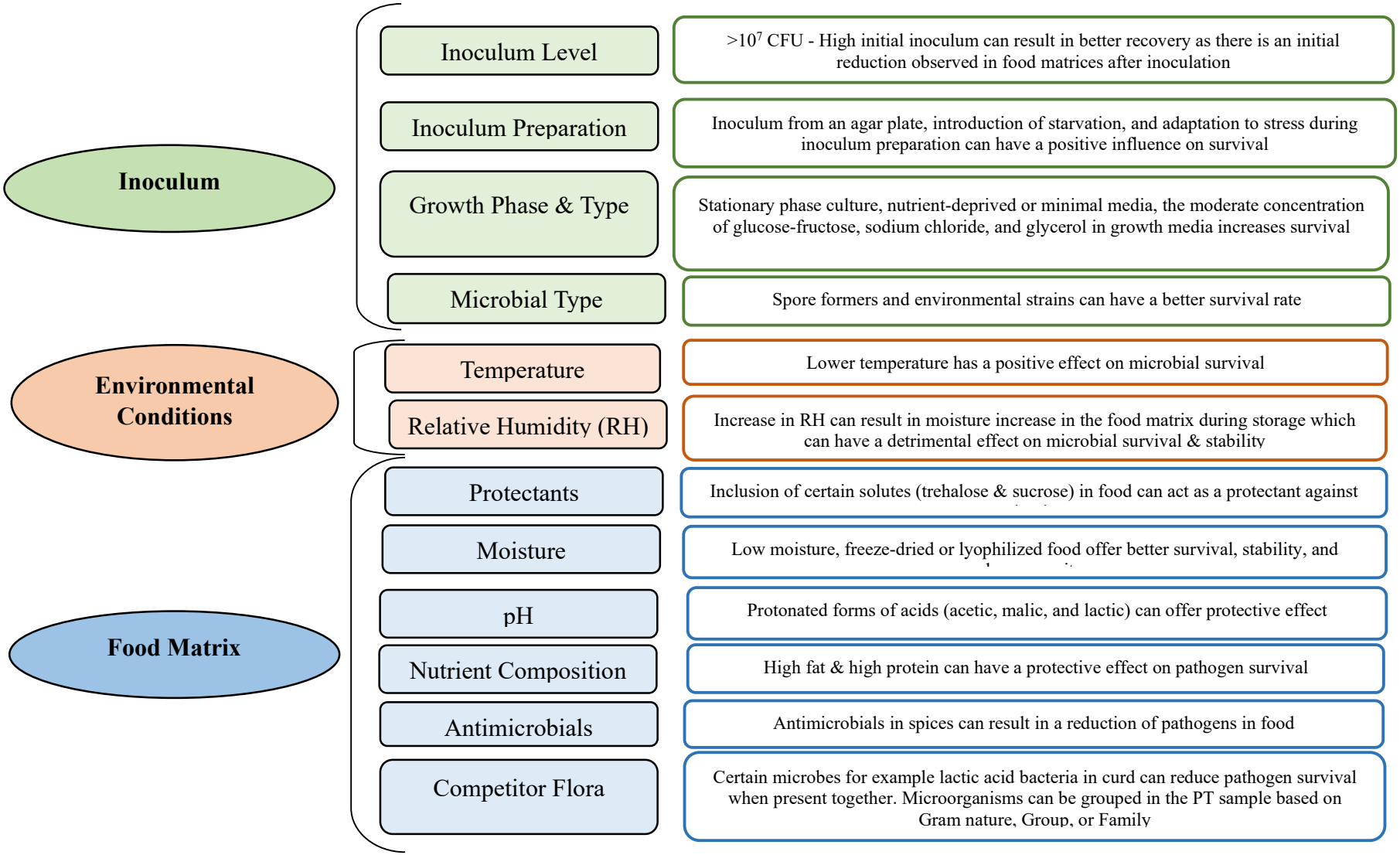


Figure 3. Factors to be considered during development of PT samples for better survival of food pathogens

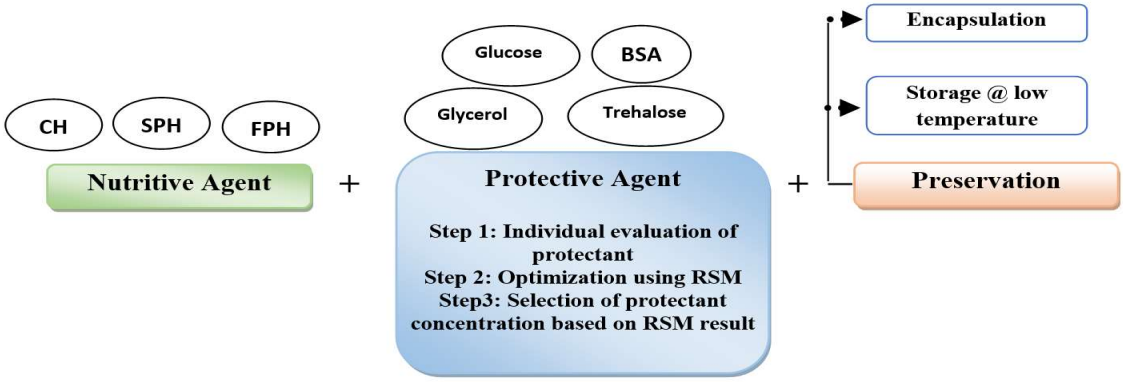
CHAPTER 3

HYPOTHESIS

3.0 Hypothesis

In the current scenario, RM with and without food matrices are constantly required in microbiology laboratories involved in food testing. The major category of RM used routinely in food testing microbiology laboratories are qualitative RM which are provided by agencies like the American Type Culture Collection (ATCC), Microbial Type Culture Collection (MTCC), and National Collection of Type Culture (NCTC). These agencies characterize and maintain cultures in their repositories, which have traceability established to internationally accepted culture collection (17). For example, most of the MTCC cultures are characterized for equivalence to ATCC. They are made available in different formats, e.g., on nutrient agar slants, as lyophilized or freeze-dried in vials, capsules, or plastic loops. Such strains are procured by reference material providers and further used to develop assigned value RM or PT samples. Laboratories typically acquire RM from culture collections directly or through deposits made by researchers, which are then revived and tested for specific parameters to maintain consistency. Various methods are followed by laboratories for maintaining cultures, such as using 5 – 10% glycerol or 15% v/v glycerol combined with 5% sucrose and inulin with lyophilization. One of the drawbacks of using qualitative RM is their inability to predict the accuracy and precision of the testing process. This limitation is overcome by using quantitative or assigned value RM, as we know the exact range of micro-organisms present in quantitative RM. Additionally, quantitative RM can be used for method validation, method verification, and skill development of microbiologists. However, the use of quantitative RM are limited due to its restricted availability in developing countries and cost-effectiveness due to its single use. In India, there are no commercial manufacturers involved in producing quantitative RM, and less than 1.4% of scientific literature available in Scopus pertains to RM and CRM in ‘Microbiology & Immunology’. Consequently, to address this deficiency and enhance the reproducibility of microbiological testing laboratories, urgent research is needed in the development of quantitative RM for the most tested microorganisms in food testing labs, namely *E. coli* and *Salmonella* spp.

The present study aims to develop a cost-effective formulation for creating stable and homogeneous quantitative RM of *E. coli* and *Salmonella*. Various nutritional and cryoprotective agents will be assessed to identify the best combinations that ensure the development of validated RM. The preservation technique of encapsulation will be used in conjunction with storage at low temperatures to increase the shelf life of the produced quantitative RM. Based on the literature review, protein hydrolysates of casein (CH), soy (SPH), and fish (FPH) will be evaluated as nutritive agents, along with glucose, glycerol, trehalose, and bovine serum albumin as cryoprotectants. Further, response surface methodology will be employed following individual analysis of nutritive and cryoprotective agents to minimize the experimental runs and identify any synergistic or antagonistic effects on the target microorganisms. Figure 4 provides a schematic representation of the components used in developing the quantitative reference material formulation, and figure 5 outlines the steps involved in quantitative reference material development, followed by the inclusion of food matrix and comparison with available commercial reference material. The inclusion of SMP as a food matrix will be studied to create PT samples after the development of quantitative RM.



Casein hydrolysate (CH), Soy protein hydrolysate (SPH), Fish protein hydrolysate (FPH), Bovine Serum Albumin (BSA)

Figure 4. Schematic representation of components used in the development of quantitative reference material formulation

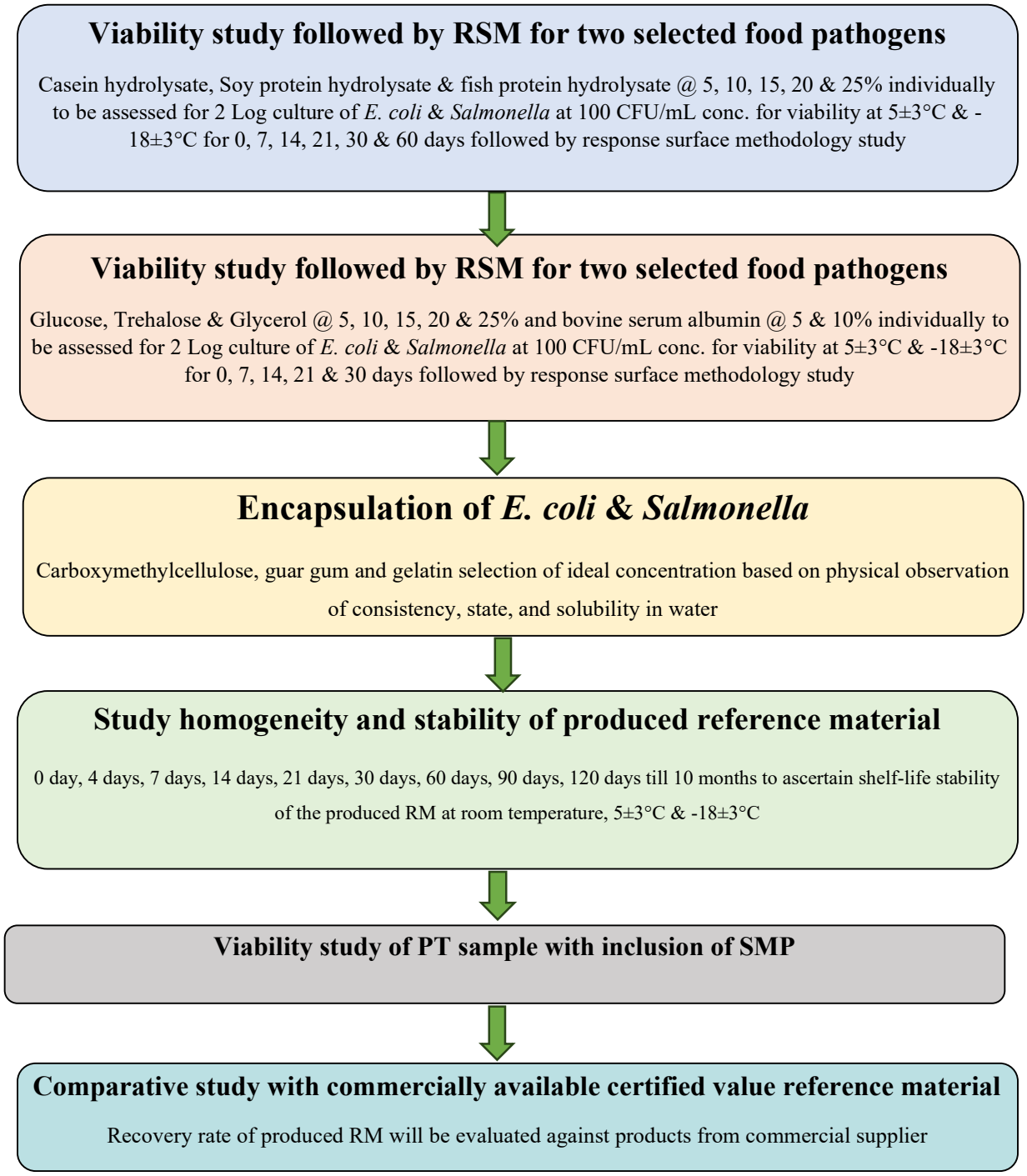


Figure 5. Steps involved in quantitative reference material development followed by inclusion of food matrix and comparison with available commercial reference material

CHAPTER 4

OBJECTIVES

4.0 Aims & Objectives

4.1 Background

A simple keyword search for ‘reference material’ and ‘certified reference material’ in Scopus yielded 14,068 documents. Of these, 12,311 documents pertain to the subject areas of chemistry, biochemistry, genetics, and molecular biology. In contrast, only 181 documents i.e., approximately 1.4% of the total literature, are related to immunology and microbiology. Therefore, it can be concluded that RM for chemical testing has been more extensively developed than those for microbiological testing, largely due to the greater ease of spiking and recovery compared to microorganisms. Assigned value or quantitative or known value cultures are used extensively in pharmaceutical industries to demonstrate the accuracy of their readings in microbiology. In the EU and the US, the same practice is conducted in food testing laboratories as well. These quantitative RM can shed light not only on our testing skills but also on our testing methodology and process.

According to the IDA list, 39 institutes across 26 countries provide qualitative RM for food microbiology testing. However, globally, only three institutes, one each in France, Germany, and the UK, offer quantitative microbiological RM. In India, quantitative microbiological RM are primarily imported from the US or the EU. The extensive R&D conducted by foreign manufacturers often leads to patented technologies, which increase costs. Additionally, customs duties further contribute to the high expense, making the regular use of quantitative RM in microbiology labs costly and economically impractical. Consequently, the development of quantitative microbiological RM could tap into unexplored markets in developing countries due to their current limited availability. This study can support the ‘Make in India’ mission of NPL and provide guidance to RM manufacturers in India to produce quantitative CRM in microbiology.

4.2 Objectives

Looking at the aim of developing quantitative RM, the objectives mentioned below were undertaken to achieve the proposed study:

1. Development of hydrophilic tube of quantitative RM using cryoprotectants with *E. coli* & *S. enterica* as target analytes.
2. Develop quantitative RM with food matrix (skimmed milk powder) and compare viability with direct inoculation and in tube format.
3. Validation of developed quantitative RM tube with commercially available quantitative RM.

CHAPTER 5

MATERIALS & METHODS

5.0 Materials & Methods

The research work was carried out to develop quantitative RM by identifying nutritive and protective agents along with the application of preservation techniques of encapsulation and low temperature. After the development of quantitative RM of *E. coli* and *S. enterica*, PT samples, including SMP as a food matrix were formulated. As a last step to assess the performance of the developed quantitative RM, commercially available RM containing similar target analytes were tested, and their performances were compared. The experimental steps are elaborated in the form of a flowchart in figure A2.

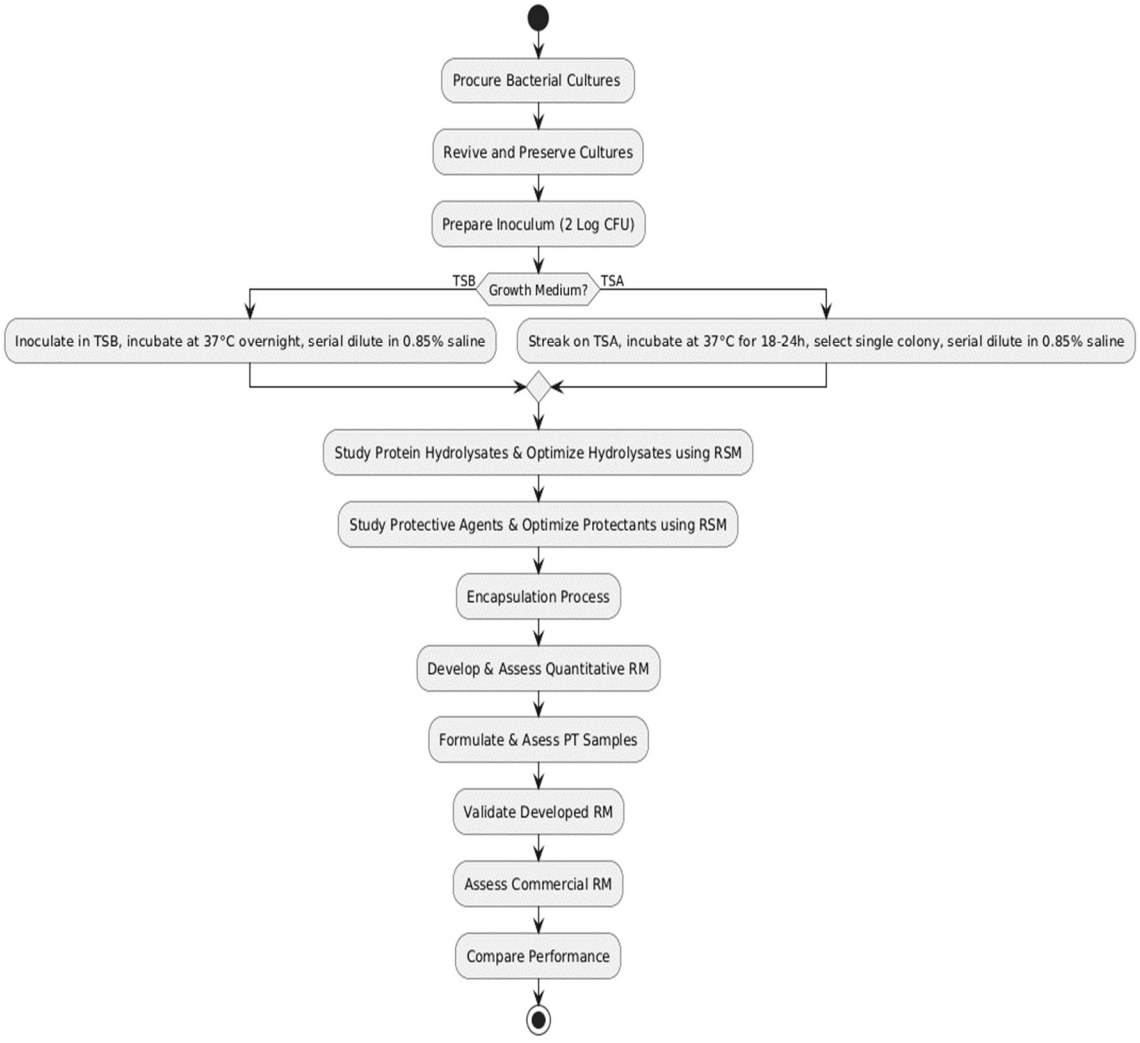


Figure A2. Flowchart summarizing experimental workflow

5.1 Bacterial cultures for the development of quantitative RM

5.1.1 Procurement of Culture

The Microbial Type Culture Collection and Gene Bank (MTCC) strain of *E. coli* MTCC 1610^T and *S. enterica* subspp. Arizonae MTCC 660^T was procured from the Institute of Microbial Technology (IMTech), Chandigarh (Figure 6). The MTCC was recognized by the World Intellectual Property Organization, Geneva, Switzerland as an IDA under the Budapest Treaty and provides microbial culture traceable to reputable culture collections which include ATCC, NCTC, German Collection of Microorganisms and Cell Cultures (DSM). The MTCC is an affiliate member of the World Federation for Culture Collections (WFCC) and is registered with the World Data Centre for Microorganisms (WDCM).

E. coli MTCC 1610^T is equivalent to NCTC 9001, ATCC 11775, CN 4382 & DSM 30083 whereas *S. enterica* subspp. Arizonae MTCC 660^T is equivalent to NCTC 8297 & ATCC 13314 in a lyophilized or freeze-dried state. Both the selected strains are type strains, wherein the MTCC has conducted viability and extensive identification tests to ascertain the culture type and biochemical response.

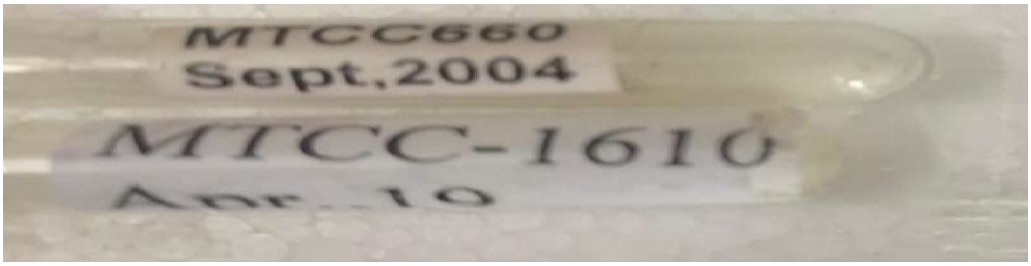


Figure 6. Cultures received from Microbial Type Culture Collection, IMTech (Chandigarh)

5.1.2 Culture revival & preservation

Resuscitation of the procured strains was performed as per the instructions received from IMTech, on nutrient broth at 25°C following an incubation of 24 h. The ampoules were opened carefully due to the presence of a vacuum, and 70% ethanol was used to disinfect

the ampoules. A sharp file was used to make a groove at mid-way of the cotton wool present inside the ampoule, and the glass ampoule was snapped open by breaking its tip using a sterile cloth. The cotton wool was removed from the ampoule, and 0.3 mL of the nutrient broth was added to resuspend the lyophilized granules. After 20 minutes, the suspension was transferred into 10 mL of the nutrient broth. After the revival of lyophilized cultures, quality checks were performed using trypticase soy agar (TSA), MacConkey agar, and xylose lysine deoxycholate (XLD) agar. After ascertaining the quality of revived cultures, 10% glycerol stocks were prepared for long-term usage and stored at -18±3°C. Sterile glycerol was added to resuscitate cultures present in the nutrient broth at 10% concentration. The prepared glycerol stock (0.5 mL) was further dispensed in sterile Eppendorf tubes, which were preserved by storing at -18±3°C until further use.

5.1.3 Inoculum preparation for study

For the preparation of 2 Log CFU culture, *S. enterica* and *E. coli* were inoculated in tryptone soy broth (TSB) and incubated overnight at 37°C, followed by serial dilution. The last three serial dilution tubes (10^{-7}, 10^{-8}, and 10^{-9}) were used for estimating the culture density. After estimating the titer, the appropriate serial dilution tube was used for the study (59,143). Further, the effect of inoculum type on the growth and survival of the test organism was studied by reviving the 10% glycerol stock on trypticase soy agar (TSA) instead of TSB. In the earlier method of inoculum preparation, the growth medium was TSB, and, after revival at 37±1°C for 18 - 24 h, the TSB inoculum was subjected to serial dilution in 0.85% saline, and the desired serial dilution was used for further inoculation in protein hydrolysates and protectants study.

For agar-grown inoculum, TSA was used as a growth medium for the revival of *S. enterica* and *E. coli* present in 10% glycerol stock solutions. The bacterial culture was streaked on TSA and incubated at 37±1°C for 18 - 24 h. A single colony was selected for serial dilution in 0.85% saline to estimate the titer, and the desired dilution tube was used for further inoculation in FPH. Using agar instead of broth for inoculum preparation introduced additional environmental stress to previously stressed cultures. These cultures,

preserved in glycerol under freezing conditions, were evaluated to determine the survival of *S. enterica* and *E. coli* due to the substitution of agar as the growth medium (15).

5.2 Protein hydrolysates used in the study

Peptones are classified based on their source, and the four major categories are meat (e.g., tryptose and gelatin), vegetable (e.g., soy and pea), milk-derived (e.g., casein and whey), and mycological (e.g., yeast and fungi) (47). CH, FPH, and SPH were selected for this study to represent the three major categories of peptones. The nitrogen content of plant- and animal-derived protein hydrolysates ranges from >7% to 15% and is mostly dependent on the source and method of hydrolysis employed (thermal, acidic, alkaline, or enzymatic) (144–146). The total nitrogen content from proteins estimated with the Kjeldahl method based on commercial manufacturer data for CH and SPH were ≥ 8% and ≥ 12%, respectively. *Stolephorus indicus* was used to produce FPH, which has an average of 12±1% of total nitrogen content from protein (147)

5.2.1 Fish protein hydrolysate

FPH was provided by ICAR – Central Institute of Fisheries (CIFT), Kochi, and was processed using Indian anchovy (*Stolephorus indicus*). FPH was received in an airtight container was stored until further use. FPH produced by CIFT was a light brown colored powder containing <10% moisture and a total nitrogen content of 13.5±0.5%.

5.2.2 Casein hydrolysate

Technical-grade CH obtained from Loba Chemie (Catalogue no. 02575-500G) was a light brown powder with an ash content of 20.80%, sodium chloride content of 15.71% estimated by silver nitrate titration method, and a total nitrogen content of 12.7 %.

5.2.3 Soy protein hydrolysate

SPH obtained from Merck (Sigma catalogue no. S1674-100G) was a beige-colored powder derived from the hydrolysis of soybeans (*Glycine max*). SPH is soluble in water in the

presence of a slight haze containing 4% moisture, 8% ash, 9% amino nitrogen, and 13% total nitrogen.

All three hydrolysates were sterilized using a boiling water bath for 15 minutes and autoclaving at 121°C for 5 minutes. Each hydrolysate was studied individually on both microorganisms by preparing 5, 10, 15, 20, and 25% concentrations in saline. Viability of the microorganisms was assessed in the hydrolysates using TSA at 37±1°C for 18 - 24 h for 0, 7, 14, 21, 30 & 60 days.

5.3 Protectants

Preservation techniques, such as cold temperature storage and encapsulation, are effective tools for controlling microbial cell growth. These techniques are complemented by the inclusion of protective agents to prevent cell damage, as noted by various researchers in the past (30,52). For this study, glycerol, glucose, trehalose, and bovine serum albumin (BSA) were selected due to their proven protective properties (33). Each of these four protective agents was individually assessed on both the microorganisms at two different temperatures, 5±2°C and -18±3°C.

5.3.1 Glucose

Anhydrous glucose (extra pure) of S.B Laboratories Chemicals (CAS No. 50-99-7) having a molecular weight of 180.16 g/mol, white colour in appearance and completely soluble in water was used as a protectant in this study.

5.3.2 Glycerol

Glycerol (purified) of S.B Laboratories Chemicals (CAS No. 56-81-5), a colorless liquid having a molecular weight of 92.09 g/mol, and 98% in assay content, was used as a protectant.

5.3.3 Trehalose

D-(+)-Trehalose dihydrate was procured from an authorized distributor of Merck (Sigma catalogue no. 90210-50G). It was a white colored powder of 100% purity as tested by the

HPLC method, specific rotation of 177.7 degree, and passes trace metal conformity requirement with no presence of glucose as estimated by enzyme assay.

5.3.4 Bovine Serum Albumin

Bovine albumin fraction V powder was procured from Loba Chemie (Catalogue no. 02230-10G). It was cream colored crystalline powder in appearance, with 100% purity estimated by assay and 16% nitrogen content. The pH value of 1% solution in water was 7.47 with 0.029% moisture.

Glucose, trehalose, and glycerol were sterilized by autoclaving at 121°C for 15 mins, whereas BSA was sterilized using a 0.2-micron membrane filter. Glucose, trehalose, and glycerol were studied individually on both microorganisms by preparing 5, 10, 15, 20, and 25% concentrations, whereas BSA was studied at 5 and 10% concentrations prepared in saline. The viability of the microorganisms was assessed in the protectants using TSA (37±1°C for 18 - 24 h) at 0, 7, 14, 21, and 30 days.

5.4 Media & Diluents

TSB and TSA were used in the inoculum preparation for the individual study of hydrolysate and protective agents. Trypticase or tryptone soya medium is a versatile medium and is mostly used as a revival medium due to its non-selective nature.

5.4.1 Trypticase Soya Broth

TSB (composition provided in Appendix 1), commonly known as soybean casein digest medium is used as a general-purpose media for the growth of aerobes and fungi. TSB is a highly nutritious media used for the cultivation of a wide variety of microorganisms.

5.4.2 Trypticase Soya Agar

TSA (composition provided in Appendix 1) was prepared by adding agar at 1.5% concentration in TSB.

5.4.3 Diluent

Different concentrations of hydrolysates and protective agents were prepared in saline to prevent osmotic imbalance of the target microorganisms to be studied without interfering with the study results. Saline (composition provided in Appendix 1) was used as the diluent.

5.4.4 Selective media for culture quality check

MacConkey agar (composition provided in Appendix 1) and XLD agar (composition provided in Appendix 1) were selected for quality check of *S. enterica* and *E. coli*, as both the microbes provide specific type of colonies on these media.

5.4.5 Nutrient broth

Nutrient broth (composition provided in Appendix 1) and nutrient agar (composition provided in Appendix 1) were used as a growth medium for resuscitation of freeze-dried or lyophilized cultures received from IMTech, Chandigarh.

5.5 Encapsulation

Cryopreservation and lyophilization have been the most widely used techniques for microbial preservation, but they cannot be applied to all microorganisms and have various limitations affecting cellular viability (148). To address the issues of cell protection in adverse environments of the culture medium and cultivation conditions, cell immobilization techniques have been extensively used in bioprocess. The immobilization of probiotic microorganisms on different support media has also been studied, leading to prolonged cell viability (149,150). After an initial assessment, the encapsulation methodology was employed, including target culture, nutritive agent, and protective agents to form the quantitative RM.

5.5.1 Encapsulating agents

Gelatin powder was sourced from Loba Chemie (Catalogue no. 03920) and had a faintly yellow translucent appearance in granules with 9.29% moisture content. The presence of zinc, iron and chromium conforms to the specification and was soluble in water at 1%

concentration. Carboxymethyl cellulose sodium salt (CMC) with high viscosity (Loba Chemie Catalogue no. 02530) was used for the encapsulation study. It was a white colored powder with 4.17% moisture and conforms to the specification requirements of heavy metals, including lead, arsenic, and iron. Guar gum (HiMedia Catalogue no. GRM1233) was selected as a thickening agent for the encapsulation process. It formed a colloidal solution with water and had a beige color in powder format with 10.88% moisture content.

5.5.2 Physical assessment

All three encapsulating agents were studied initially for water solubility and the time for drying of a formed disc/pellet by the drop method. CMC (0, 1 & 2%), gelatin (1,2,3 & 4%), and guar gum (0,1,2,3 &4%) were studied at different concentrations by mixing using a magnetic stirrer at 45°C for 30 min to assess consistency. Later, the mixtures were used to form pellets, which were dried in a laminar flow for 3 h and checked for the state or form of the pellets. Solubility of the dried pellets were assessed by mixing in distilled water and checked for residue.

5.5.3 Process

Based on the physical assessment, encapsulating agents and their respective concentrations were selected for the encapsulating process. The encapsulating agents were heated at 45°C and mixed using a magnetic stirrer with selected nutritive and protective agents. Later, overnight-grown culture on TSA is added at a concentration to lead to 2 Log culture in each Eppendorf tubes to make 1.0 mL of quantitative RM.

5.6 Response surface methodology software

Design Expert V 22.0.0.1 (US, Stat-Ease Inc.) software was used for optimization studies employing RSM. RSM, a multivariate optimization strategy, was used to reduce reagent or consumable spending, along with a reduction in laboratory work owing to the optimization of the required experimental runs (151). The selection of RSM was based on the opportunity to significantly reduce experimental runs without compromising the study, as it establishes a correlation between the different variable factors and extrapolates to provide

the relationship (if present) between the different factors in the study. The RSM software used a quadratic model, and accuracy was determined using analysis of variance (ANOVA).

5.6.1 Response surface methodology study for protein hydrolysates

The response from the RSM study was tested for a quadratic model utilizing the BBD, as there were three variable factors involved: CS, SPH, and FPH in the optimization study of hydrolysates. The lowest level factor for each of the protein hydrolysates was set at 0, the mid-level factor at 5%, and the highest factor at 10%; the coded value for the lowest level factor, mid-level factor, and the highest level factor was -1, 0, and +1, respectively. The coded factor range and corresponding levels are presented in Table 4.

Table 4: Coded experimental factor range and levels for protein hydrolysates in optimization study

Coded Factors	Actual Factors	Range and levels		
		−1	**0**	**+1**
A	Casein hydrolysate	0%	5%	10%
B	Soy protein hydrolysate	0%	5%	10%
C	Fish protein hydrolysate	0%	5%	10%

Refrigeration (5±2°C) and freezing temperatures (-18 ± 3°C) were used for storage, and after regular intervals, the viability was assessed using TSA in triplicate. The two types of variables included in this multivariate study were responses and factors. The responses were the dependent variables, and the factors had three levels. Based on individual studies on each protein hydrolysate, the factors were finalized at three concentration levels. In our study, more than one response was observed, as the response was measured in the form of survival/viability at consecutive intervals of 0, 7, 14, and 28 days. Employing BBD for RSM was beneficial, as it allowed the estimation of parameters using a quadratic model, sequential design build-up, application of blocks, and model lack-of-fit detection (151).

The correlations between the variables in the quadratic model and the three variables is represented by Eq. 1 whereas the correlation between variables in a linear model and the three variables is represented by Eq. 2. The coefficient estimate represents the expected change in response per unit change in the factor value when all the remaining factors are held constant. The intercept in the orthogonal design is the overall average response of all runs. The coefficients are adjusted around that average based on the factor settings.

$$Y = Intercept + x_1(A) + x_2(B) + x_3(C) + x_4(AB) + x_5(AC) + x_6(BC) + x_{17}(A)^2 + x_8(B)^2 + x_9(C)^2 \quad (1)$$

$$Y = Intercept + x_1(A) + x_2(B) + x_3(C) \quad (2)$$

where Y = response value (survival rate), A = casein hydrolysate, B = soy protein hydrolysate, C = fish protein hydrolysate, and x_1– x_9 = coefficient estimates of the respective factors.

5.6.2 Response surface methodology study for protective agents

The effects of each protective agent (glucose, glycerol, trehalose, and bovine serum albumin) were individually studied on *E. coli* and *S. enterica* at concentrations ranging from 5 – 25%. This individual study was used to identify the optimal concentration range for each protectant, and further optimization was carried out using the Response Surface Method (RSM). The software used for the RSM study was Design Expert™ (V 22.0.0.1) from Stat-Ease Inc., USA. The RSM software uses a quadratic statistical model and utilizes Analysis of Variance (ANOVA) for the assessment and verification of the proposed model by the software. For the design of the experiment, the BBD was used, as the experiment involved more than three factors. The statistical model codes the variable factors and the selected concentration in a three-level factorial design, which is -1, 0, +1, where 0 represents the center point (64). The experimental details of the coded value for variable factors and the three factorial experimental levels are detailed in Table 5.

Table 5: Coded experimental factor range and levels for protectants in optimization study

Coded Factors	Actual Factors	Range and levels		
		−1	**0**	**+1**
A	Glucose	5	10%	15%
B	Glycerol	5	10%	15%
C	Trehalose	5	10%	15%
D	BSA	0	2.5%	5%

For a four-factor variable (protectants) involving three factorial levels of BBD design, the RSM software resulted in a total of 29 experimental runs. The correlation between the variables in a quadratic model with four variables is represented by Eq. 3 whereas the linear model is represented by Eq. 4. The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. Response value was observed at fixed intervals of 7 days up to 28 days and a total of five responses were generated for each experimental run including that of Day 0, when the inoculum was added.

$$Y = Intercept + x_1(A) + x_2(B) + x_3(C) + x_4(D) + x_5(AB) + x_6(AC) + x_7(AD) + x_8(BC) + x_9(BD) + x_{10}(CD) + x_{11}(A)^2 + x_{12}(B)^2 + x_{13}(C)^2 \quad (3)$$

$$Y = Intercept + x_1(A) + x_2(B) + x_3(C) \quad (4)$$

were Y = Response Value (Survival Rate); A = Glucose; B = Glycerol; C = Trehalose; D = BSA & x_1 – x_{13} = coefficient estimate of respective factors

5.7 Study of developed quantitative reference materials

Three batches of quantitative RM were produced and was measured for homogeneity & stability as per ISO 33405:2024 and were stored at three different temperatures of room

temperature (RT) (31±5°C), refrigerator (5 ± 2°C) & freezer (-18 ± 3°C). Since the batch size was <100 units for each storage temperature, homogeneity needs to be assessed for a minimum of three RM units or 10% of the batch size. A total of fifteen RM units were assessed for each batch in triplicate at each selected interval of time, and the average mean was calculated for each of these 15 readings obtained. Hence, the total number of 90 RM units (30 RM units per batch) was measured over a period of 150 days (5 months) for of produced RM stored at -18 ± 3°C, which is over the requirement of 10 % of the batch size (65). Viability testing of the developed quantitative RM was carried out using TSA at 35±1°C for 24 h. Pour plate technique was used instead of the spread plate technique as the former is the most used technique in food, cosmetics, and pharma industries due to ease and time saving. The disadvantage of thermal shock associated with pour plate was addressed by using temperature-controlled water bath (45±1°C) for tempering of media, and the media was added to the sterile petri plate after the addition of the test sample, and the added media was mixed by swirling. For the viability testing, five RM tubes from each batch were removed from their respective stored temperature and stand at room temperature (31±5°C) for 30 mins to thaw, followed by the addition of 1 mL sterile saline diluent containing 0.85% sodium chloride and gently mix the content, avoiding agitation or bubble formation. After 30 mins post addition of saline, gently mix the content, and remove 1 mL of the content, and plate in triplicate on TSA. Standard deviation (SD) was calculated individually for individual batches for repeatability and collectively for all three batches to evaluate reproducibility. An analysis of variance (ANOVA) was conducted on the viability readings to calculate the p-value, F-value, and mean Log variation. This test aimed to assess whether the produced quantitative reference materials (RM) exhibit homogeneity. SD was monitored for the ten readings obtained from testing of five RM units in triplicate at each selected interval of time from three different batches of the produced quantitative RM, the SD of the produced RM should be ≤ 0.35 Log_{10} based on the binomial probability of detecting 0 defective units when 10% of the true incidence of defective unit in a lot (152,153).

5.8 Development of proficiency testing sample

As a further extension of the application of quantitative RM, it needs to be determined whether the developed RM can be used along with the food matrix as a Proficiency Testing (PT) sample. As per standards (ISO 17025:2017), accredited microbiology laboratories need to use certified reference materials (CRM) and enroll in the PT scheme to ensure the competency of lab microbiologists. PT sample is a quantitative RM with a food matrix. Various researchers like (4,9,69,154) have mentioned the usage of SMP as a food matrix in the development of qualitative or quantitative RM. Also, SMP as a food matrix represents the dairy food segment; hence development of a PT sample with a dairy food matrix will have a larger application and usage. SMP was added in the developed quantitative RM to understand the impact of SMP on the performance of the developed quantitative RM and to evaluate the possibility of creating a PT sample. The impact of the inclusion of SMP was studied by conducting a recovery or viability study by adding SMP in ten quantitative RM units developed of each target analyte using TSA at 37±1°C for 18 - 24 h. The data was subjected to z-score estimation, which is calculated as per Eq. 5 for ten PT samples tested for each microorganism. z-score is used to understand the normal distribution of the target analyte in the food matrix. Hence calculated z-score should be within the range of +2 and -2.

$$z\ score = \frac{x - \mu}{\sigma} \quad (5)$$

where x = observed or recovery value of an individual PT sample, μ = mean of the quantitative RM sample, and σ = standard deviation observed for a set of 10 recovery values of the PT sample.

A chi-square test was applied to the recovery data to determine whether the proposed null hypothesis ***"There is no impact of SMP on the actual value of quantitative RM results"*** holds. Chi-square was calculated as per Eq. 6 and the calculated Chi-square value should be less than the tabulated Chi-square value at a 5% level of probability.

$$\chi^2 = \sum \frac{(Observed\ value - Exp\ \ \ \ \ value)^2}{Expected\ value} \quad (6)$$

where χ^2 = Chi-square and $\sum$ = Sum

5.9 Validation of the developed quantitative reference materials with the commercial reference material

This step was conducted to understand the performance of developed quantitative RM with respect to the commercially available quantitative RM.

5.9.1 Assessment of commercial reference material

Commercially available products from Sigma Aldrich (Vitroids^{TM}) were procured for *E. coli* WDCM 00013 (catalogue no. VT000133) and *S. enterica* Enteritidis WDCM 00030 (catalogue no. VT000303), containing ten samples for each microorganism. The samples were tested on TSA at 37±1°C for 18 - 24 h and were assessed for standard deviation to be within Log_{10} 0.35 along with conformity to the acceptable range mentioned in the certificate of analysis.

5.9.2 Validation study of developed quantitative reference material

The performance of developed quantitative RM was studied at five institutes, of which three were commercial laboratories and two were academic institutes. The list of institutes is provided in Table 6.

Table 6*:* List and type of institutes used for validation of developed reference materials

Code No.	Institute Name	Institute Type
CL 1	Adarsh Scientific	Commercial Lab
CL 2	Autocal	Commercial Lab
CL 3	Geo-Chem	Commercial Lab
AI 1	V.E.S College (Microbiology Dept)	Academic
AI 2	LPU (School of Applied Medical Sciences)	Academic

The readings obtained from each of the institutes were subjected to z-score calculation as per Eq. 5, along with the calculation of standard deviation, which needs to be < Log_{10} 0.35.

CHAPTER 6

RESULTS AND DISCUSSION

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Singh et al. (2025) “ Development of Hydrophilic Quantitative Reference Material of *Escherichia coli* for Application in Microbiology Laboratories” Doi: 10.1007/s12161-024-02752-0

6.0 Results & Discussion

To identify nutritive agent for the formulation of quantitative reference materials, protein hydrolysates were studied individually (at 5%, 10%, 15%, 20% and 25% concentration) on *E. coli* MTCC 1610^T and *S. enterica* MTCC 660^T, leading to the maximum survival of both the micro-organisms. Type strains of *E. coli* and *S. enterica* were used in this study due to the well characterized and reliable nature of type strains, as there are very few studies available for development of quantitative microbiological RM. Usage of type strains ensures comparability, reliability, and standardization of results for replication. Many regulatory food safety authorities (FSSAI, EFSA, US FDA) across globe recommend usage of type strains for regulatory compliance to ISO. The ISO standard for the preparation, production, storage, and performance testing of culture media has listed type strains for performance testing of microbiological culture media (155). As per ISO standard for reference material manufacturing, the purity of raw material used should be characterized and standardized and since we are developing quantitative RM, type strains address the standardization of qualitative properties which include consistent biochemical reactions (1,5,65). The pour plate method was used to determine the recovery or viability rate. The effect of agar-grown, or agar propagated inoculum was also studied in the presence of FPH to understand if the growth on agar imparts any advantage in survival when compared with inoculum grown in broth. To identify and optimize the interrelationship between all three protein hydrolysates and identify the most effective concentration of protein hydrolysates, response surface methodology was conducted at 5±2°C over a storage period of 28 days. A similar approach was followed to identify effective protective agents to be included in the formulation along with the nutritive agent. As a final step encapsulation process was optimized by assessing carboxy methyl cellulose, gelatin and guar gum based on physical observation followed by viability study for determination of stability and homogeneity of produced quantitative RM. The results associated with the first objective the development of a hydrophilic tube of quantitative RM using cryoprotectants with *E. coli* and *S. enterica* as target analytes, are discussed below.

6.1 Effect of protein hydrolysates on the survival of *E. coli* and *S. enterica*

Protein hydrolysates were individually assessed at 5, 10, 15, 20, and 25% concentrations to identify their effects on the survival of *E. coli* and *S. enterica*. A culture of *E. coli* (2.85 Log_{10}) and *S. enterica* (2.16 Log_{10}) used for the study was prepared using a serial dilution of a culture grown overnight in TSB (59,143). The individual study was followed by an optimization study using RSM to investigate the presence of synergistic or antagonistic effects among the three protein hydrolysates and to identify the best concentration of protein hydrolysates leading to the maximum survival of *E. coli* and *S. enterica*.

6.1.1 Effect of casein hydrolysate on the survival of *E. coli* and *S. enterica*

The survival of *E. coli* and *S. enterica* was studied at 5, 10, 15, 20, and 25% of CH for 60 days at 5±2°C. CH exhibited *E. coli* recovery percentages of 74.86%, 61.87%, 56.21%, and 24.53% at concentrations of 5%, 10%, 15%, and 20%, respectively. However, no recovery was observed at 25% CH when stored at 5±2°C. Interestingly, after 60 days, only the 5% CH concentration showed a 53.83% recovery of *E. coli* (Figure 7). CH at 25% concentration showed an inhibitory effect on *E. coli* as there was a reduction in inoculum by 50.26%, respectively, after 7 days of storage at refrigeration conditions (5±2°C) along with a gradual decrease leading to no growth at 21 days. The fact is well supported by the previous studies reporting antimicrobial and bacteriostatic properties of bioactive peptides present in the hydrolysates towards the pathogenic micro-organisms (156–158). Most of these studies suggest that the degree of hydrolysis is directly proportional to the antimicrobial properties.

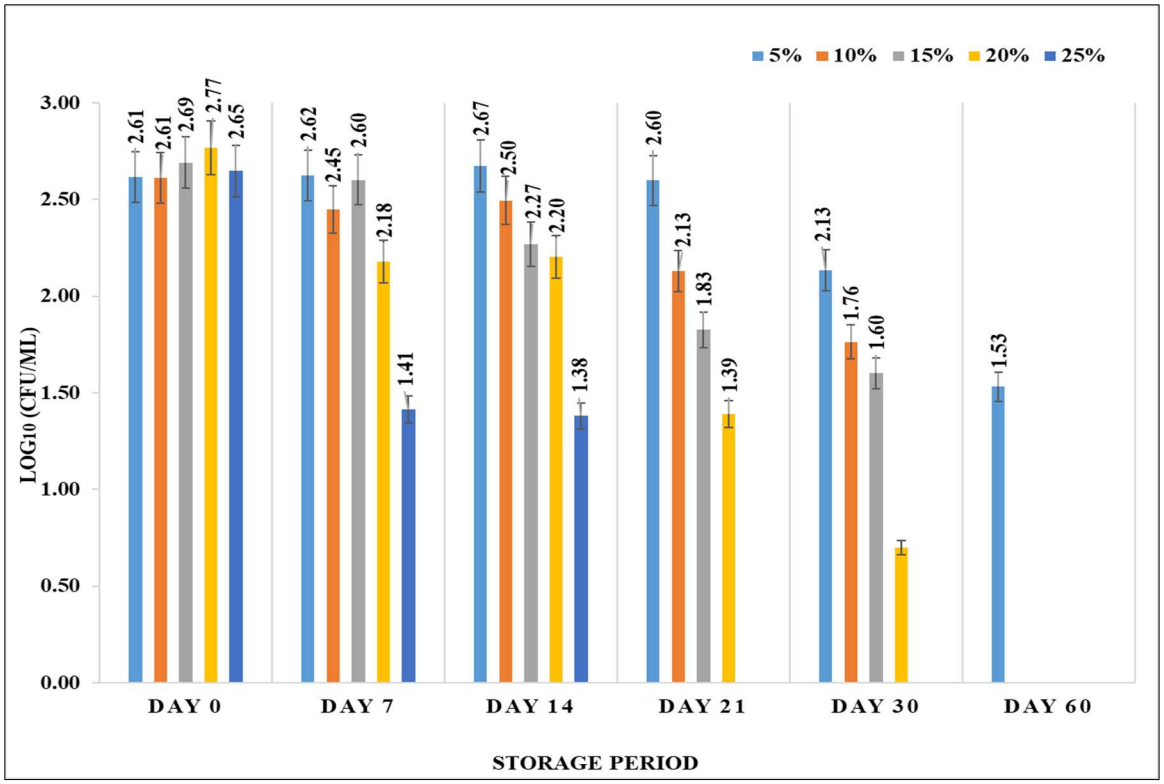


Figure 7. Survival of *E. coli* MTCC 1610^{T} in presence of 5, 10, 15, 20 & 25% casein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0,7,14,21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

The results (Figure 8) showed that 5% CH supports the growth of *S. enterica* (~59% survivors) for up to 30 days, beyond which no survivor was observed. None of the other concentrations of CH supported the survival of *S. enterica* at 5±2°C. Generally, the major protein in milk is casein (constituting 80% of the total milk proteins), and it is composed of α-, β-, γ-, and κ-casein (159). CHs and peptides derived from casein exhibit antioxidant and antimicrobial properties against foodborne pathogens, including *E. coli* and *S. enterica* (160–165). The generation time and yield of *S.* Typhimurium were dependent on the source of peptones. When used as constituents in buffered peptone water, peptones derived from yeast and gelatin produced more significant yields compared to peptones derived from casein and soy (47). These results are well supported by another study in which UV-

irradiated *S.* Typhimurium was resuscitated with CH in broth and minimal media. No survival enhancement was observed for lag phase cells in minimal media and stationary phase cells in broth and minimal media, suggesting the ineffectiveness of CH in promoting the survival of *S.* Typhimurium under stress conditions (166). Intermittent survival of *S. enterica* was observed which could result from the environmental stresses, such as low temperature and the presence of CH as the sole nutrient. These conditions may lead to a viable but non-culturable state that can be resuscitated later (167).

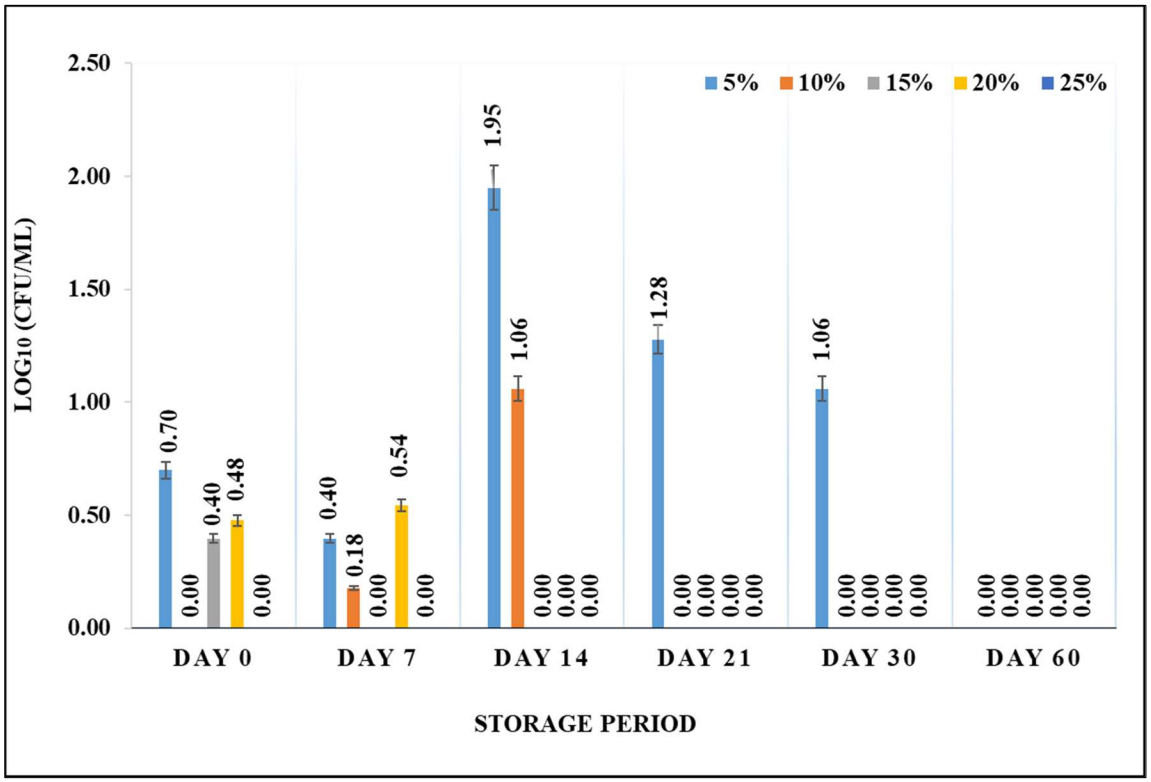


Figure 8. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in presence of 5, 10, 15, 20 & 25% casein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0,7,14,21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

Furthermore, CH did not exhibit any cryoprotective effects under freezing conditions, with no survival of *E. coli* and *S. enterica* observed at -18 ± 3°C after 7 days of

storage. Spray dried milk is one of the common food ingredients that has been studied due to several outbreaks of *E. coli* and *Salmonella* over the past few decades. In one such study, it was concluded that pasteurization is an essential step before spray drying to kill food pathogens (*E.coli* and *Salmonella*) as high moisture facilitates killing of food pathogens, whereas low moisture due to the presence of total solids in milk inhibits reduction (168). Since the individual study of protein hydrolysate involved high moisture and due to the absence of any cryoprotective properties of protein hydrolysate at freezing conditions, there was no survival observed for both the test micro-organisms.

6.1.2 Effect of soy protein hydrolysate on the survival of *E. coli* and *S. enterica*

The survival of *E. coli* and *S. enterica* was studied using 5, 10, 15, 20, and 25% concentrations of SPH at 5±2°C, and -18±3°C for a 60-day storage period. SPH at 5 and 10% concentrations showed the best recovery of 95.87 and 91.42%, respectively, whereas 15 and 20% SPH showed 75.30 and 44.04% recovery, respectively, for *E. coli* at 5±2°C for a storage period of 60 days. SPH at 25% concentration showed an inhibitory effect on *E. coli* as there was a reduction in inoculum by 58.66%, after 7 days of storage under refrigeration conditions (5±2°C), along with a gradual decrease leading to no growth on Day 60 (Figure 9). Lower concentrations of SPH (5% and 10%) were found to be most suitable concentrations for the survival of *E. coli* after 60 days when stored under refrigeration conditions. Higher concentrations of SPH (25%) had an inhibitory effect on the survival of *E. coli* after 7 days, which was observed as a decrease in the recovery. Soy proteins have often been used as a replacement for nitrogen source in the microbiological media for the production of recombinant proteins and cell growth of engineered *E. coli* (169,170). One of the drawbacks observed when replacing tryptone with soy protein is that although a rich medium containing soy protein can enhance cell growth, it may lead to a sudden decrease in *E. coli* survival during the stationary phase. This decrease could be attributed to pH changes, glycation, and an increase in the mutation frequency (171). Bioactive peptides from soy protein have been studied for their antimicrobial and antioxidant properties against *E. coli*, where an increase in antioxidant amino acids is observed with an increase in the solid concentration of soy protein isolates (172). The same

can be extrapolated that at higher concentrations of SPH, the concentration of bioactive peptides is higher leading to an inhibitory effect on *E. coli*.

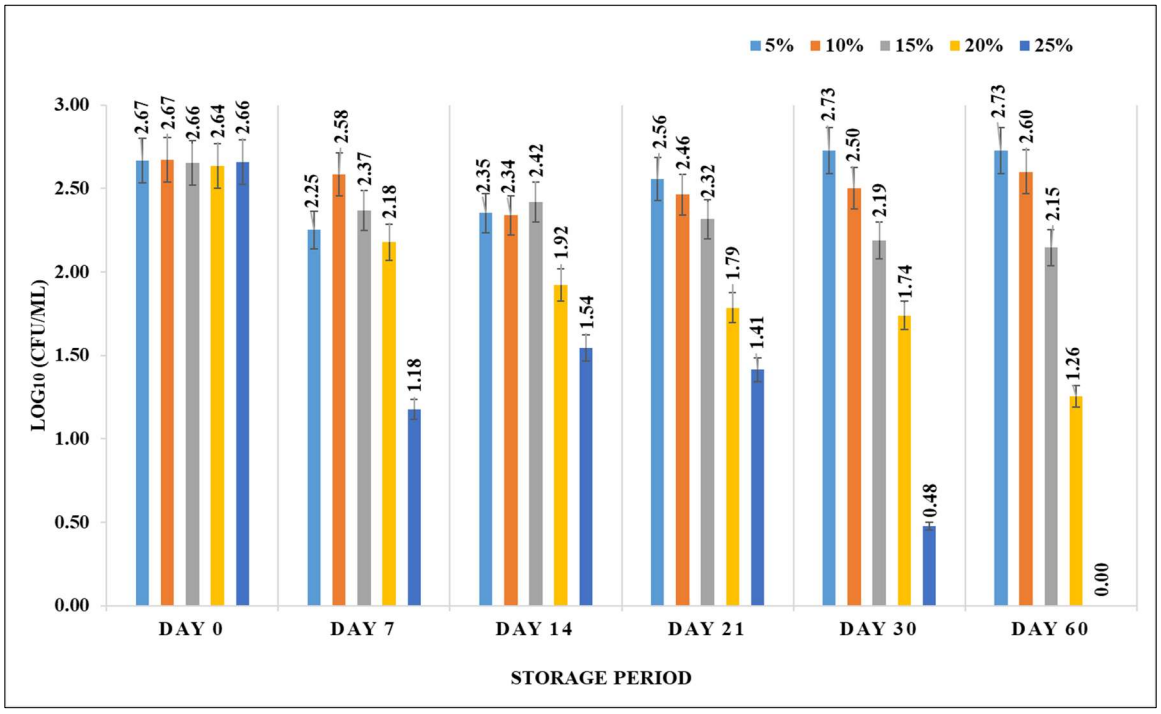


Figure 9. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% soy protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

All SPH concentrations failed to support the survival of *S. enterica* at refrigeration (Figure 10), as only 10% SPH showed 47.22% survival on Day 7, whereas 5% SPH resulted in 27.76% survival on Day 14, which reduced to no survival from Day 21 for all SPH concentrations. This pattern may result from the antimicrobial peptides present in the plant products (173). SPH is derived from soybeans (*Glycine max*) that are rich in bioactive peptides such as glycinin and β-conglycinin, which have prominent antibacterial and antioxidant effects against *S. enterica* (174). Hydrolysates from soy protein isolates inhibit the growth of *S.* Typhimurium and, in some instances, synergistic inhibitory effects can

also be seen with hydrolysates from bovine whey protein and egg protein (175). CH and SPH at concentrations of 15 – 25% were unable to support growth from Day 14 of storage, which can be attributed to the increase in the concentration of antibacterial peptides from 15% concentration onwards. This could result from an increase in a particular fraction of bioactive peptides present in CH and SPH with increasing concentrations (176,177).

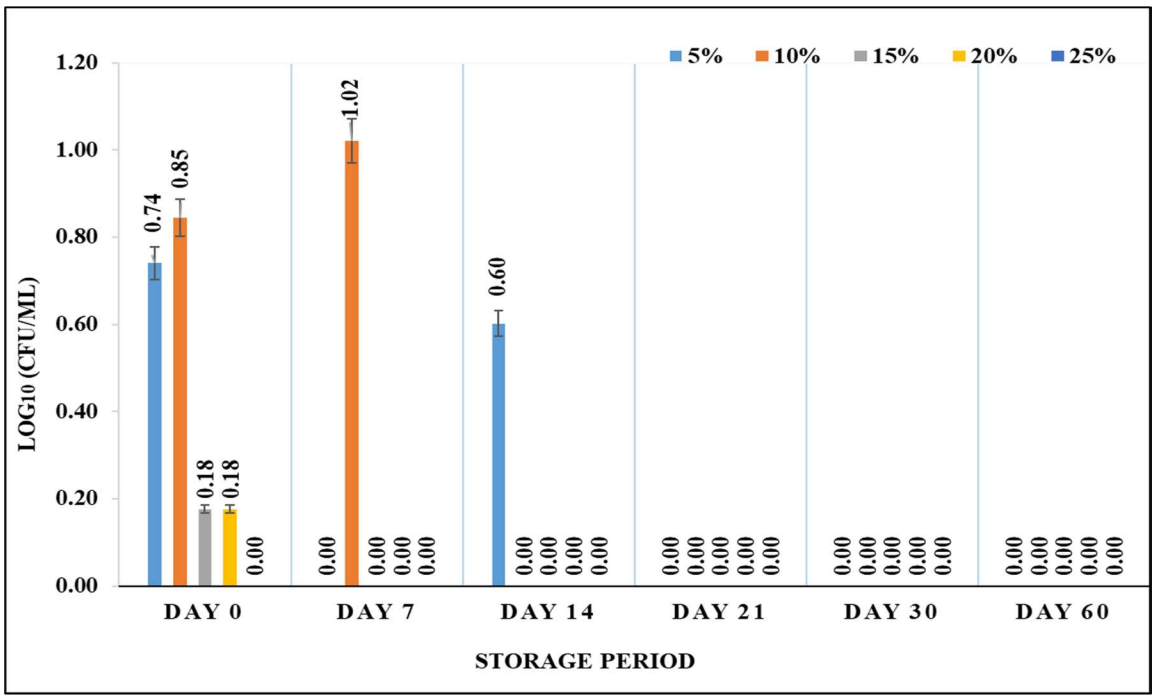


Figure 10. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in presence of 5, 10, 15, 20 & 25% soy protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0,7,14,21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

Similar to CH, all concentrations of SPH (5 – 25%) were unable to impart cryoprotection at -18±3°C, and no growth was observed from 7 days of storage period onwards for *E. coli* and *S. enterica*.

6.1.3 Effect of fish protein hydrolysate on the survival of *E. coli* and *S. enterica*

The survival of *E. coli* and *S. enterica* was studied at 5, 10, 15, 20, and 25% concentration of FPH at 5±2°C for a 60-day storage period. FPH (5% - 25%) was able to

support the survival of *E. coli* till 30 days and a linear relationship was observed between FPH concentration and survival of *E. coli*. At 5, 10 and 15% concentration of FPH, the observed recovery was 16.74, 47.10, and 68.05%, respectively, and the maximum survival was observed at 20% (78.84%) and 25% (77.30%) concentration of FPH at 5±2°C on 30 days (Figure 11). There was no survival observed on the 60th day for all the FPH concentrations. A higher concentration of FPH was found to be effective up to 30 days, but after 60 days, FPH was found to be ineffective for sustaining the growth of *E. coli*. Highly water-soluble FPH derived from hake filleting waste, containing 80% protein, was used as the sole carbon and nitrogen source in a buffer and saline composition. The growth pattern of *E. coli* when exposed to this FPH was comparable to that observed in the reference medium (Luria–Bertani medium) (178). Thus, the higher concentration of protein in FPH is capable of *E. coli* growth and survival when used as the sole nitrogen and carbon source. The sudden loss of *E. coli* viability at 60 days can be attributed to increase in glycation, mutation frequency, and metabolite buildup (171).

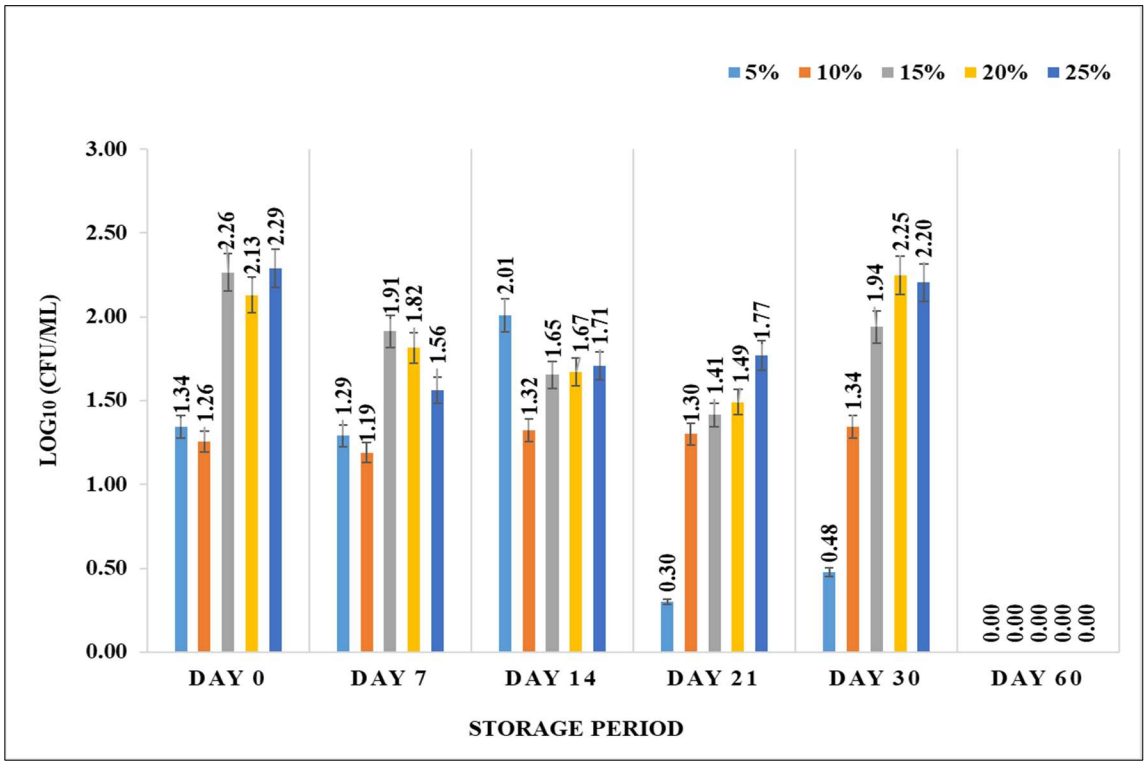


Figure 11. Survival of *E. coli* MTCC 1610^{T} in presence of 5, 10, 15, 20 & 25% fish protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

The FPH was the only hydrolysate capable of supporting *S. enterica* growth when stored at 5±2°C for 60 days. At 5, 10, and 15% concentrations of FPH and a storage temperature of 5±2°C, the bacterium showed 102.11, 113.29, and 94.42% recovery of *S. enterica*, respectively, at the end of 60 days. However, FPH concentrations of 20, and 25% were ineffective in maintaining the viability of *S. enterica* at 5±2°C after 30 days of storage (Figure 12). In a similar study conducted with 14% tuna FPH, the highest viability of *Salmonella* after freeze-drying was 67.08% (179). FPH is identified as the most effective protein hydrolysate for sustaining the growth of *Salmonella* (180). There is a study in which FPH was used in matrices along with the freeze-drying technique to create a secondary reference material for *Salmonella* (51). The peptones obtained from cod FPH have demonstrated better growth of *S. enteritidis* compared to the growth of *Staphylococcus aureus* when used in microbiological culture media (180). Higher concentrations of FPH (20 and 25%) were unsuitable for the prolonged survival of *S. enterica*. The results showed no growth of the bacterium at a concentration of 25% from Day 7 onwards, whereas a 20% concentration of FPH resulted in a decrease in survival from Day 7 onwards, with no growth on Day 14. The lack of survival of *S. enterica* at higher FPH concentrations can be attributed to an increase in the concentration of bioactive peptides, leading to an inhibitory effect on the test microorganisms (156,158).

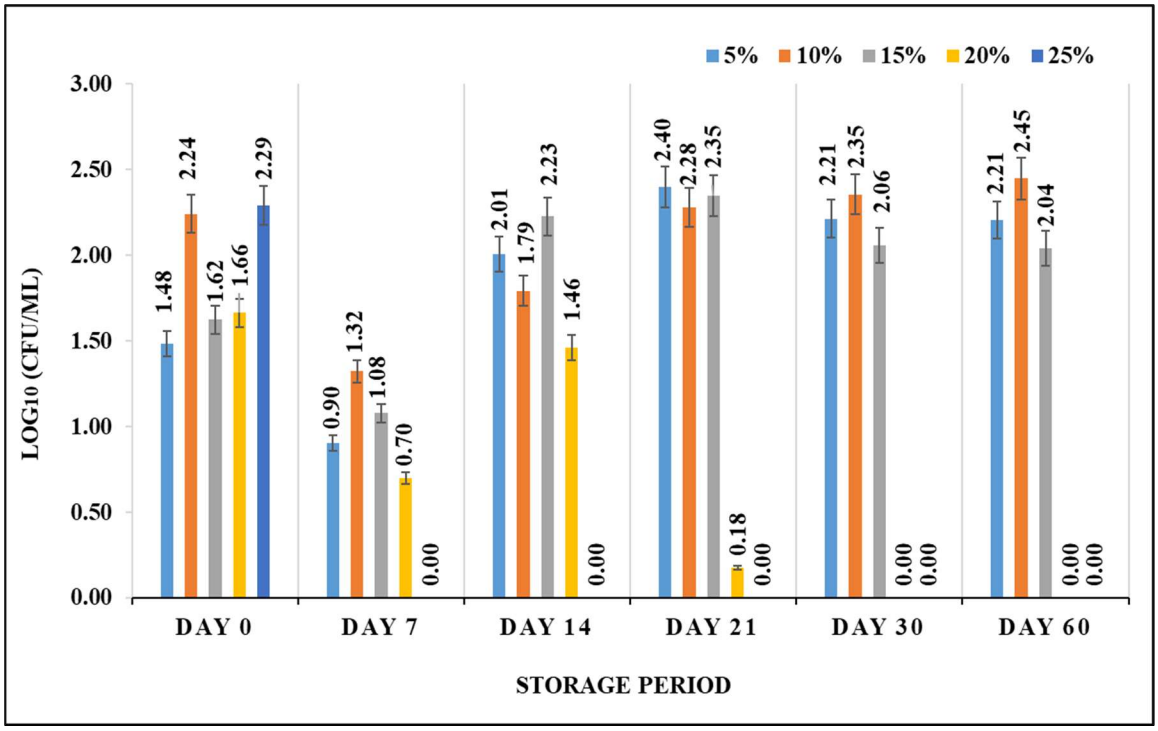


Figure 12. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in the presence of 5, 10, 15, 20 & 25% fish protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0,7,14,21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

A pattern similar to that observed with CH and SPH was also seen with FPH. Unfortunately, FPH did not provide cryoprotection to *E. coli* and *S. enterica* at -18±3°C, and no growth was observed from Day 7 onwards. While these protein hydrolysates exhibit nutritive properties for both target microorganisms, they do not possess protective properties against adverse temperatures.

6.1.4 Enhancement of survival in fish protein hydrolysate using an agar-based inoculum

In nutritionally deficient or minimal media devoid of carbon or nitrogen source, *E. coli* and *S. enterica* are subjected to environment stress, to which a quick adaptation is required. The resistance to these stressors defines the survival strategies of both the micro-organisms as they are known to survive in numerous natural, commercial, and host environments

(181,182). *E.coli* and *S.enterica* were transferred through six successive habitats ranging from dung-soil mix, excrements of snail, mice, fodder, dung, rhizosphere and phyllosphere of oat plants, where different survival rate was observed for all the different habitats (183). When *S.* Typhimurium was inoculated in manure, CFU count dropped directly post inoculation with 0.5 Log CFU gdw^{-1} (per gram manure dry weight) on Day 0 followed by increase of 0.5 Log CFU gdw^{-1} after 8 days. Similarly study with inoculation of manure with *E. coli* resulted in an instant decline of 1.5 Log CFU gdw^{-1}, followed by an increase and stabilization to 0.75 Log CFU gdw^{-1} by Day 16 (184). In another study, involving survival of pathogenic *E.coli* in pond water was studied, where a rapid decrease in *E.coli* population was observed post inoculation in water from 7 Log to 2 Log initial 14 days and enrichment was required to detect inoculated *E.coli* (185). Similarly, in our study as well we had observed initial reduction of inoculated cultures of *E.coli* and *S. enterica* on Day 0 in figure 8, 10, 11 and 12. Additionally, protein hydrolysates have been studied extensively for the production of bioactive peptides and are mostly present in varying concentrations post enzymatic hydrolysis which can contribute in the reduction of initial CFU by damaging the cell membrane (186). Adaptability to environmental stress can lead to prolonged survival, which can be induced if the inoculum is subjected to various stress conditions, including growth on an agar-based medium instead of broth (15). Hence, during inoculum preparation of *E. coli* and *S. enterica*, TSA was used instead of broth (TSB). The inoculum of *E. coli* (with a 2.76 Log_{10} initial count) prepared on agar exhibited an enhanced survival rate when inoculated into FPH, as compared to the broth-grown inoculum. This effect was observed when the samples were stored at 5±2°C for 60 days. After 60 days, agar-grown inoculum of *E. coli* showed recovery of 95.23, 90.23, and 115.13% for 5, 10, and 15% concentrations of FPH, respectively, under refrigeration conditions (Figure 13). The increase in recovery could be due to an increase in the survival capacity of the inoculum grown on agar as compared to the inoculum grown in broth. This can be attributed to an increase in the resistance and survival capacity of microorganisms under limited water activity (15). With an agar-grown inoculum of *E. coli*, at 20 and 25% concentration of FPH, there was a considerable increase in recovery along with cell growth

(135.54 and 115.24%, respectively) up to 30 days. However, on the 60th day at 5±2°C, there was a sudden decrease leading to no growth. The reason for the non-survival of *E. coli* in 20 and 25% FPH at the end of 60 days under refrigeration conditions (5±2°C) can be attributed to the ineffectiveness of the storage temperature to maintain the dormant state as the bacterial cells entered growth phase and death phase subsequently due to the development of metabolites and toxins (187).

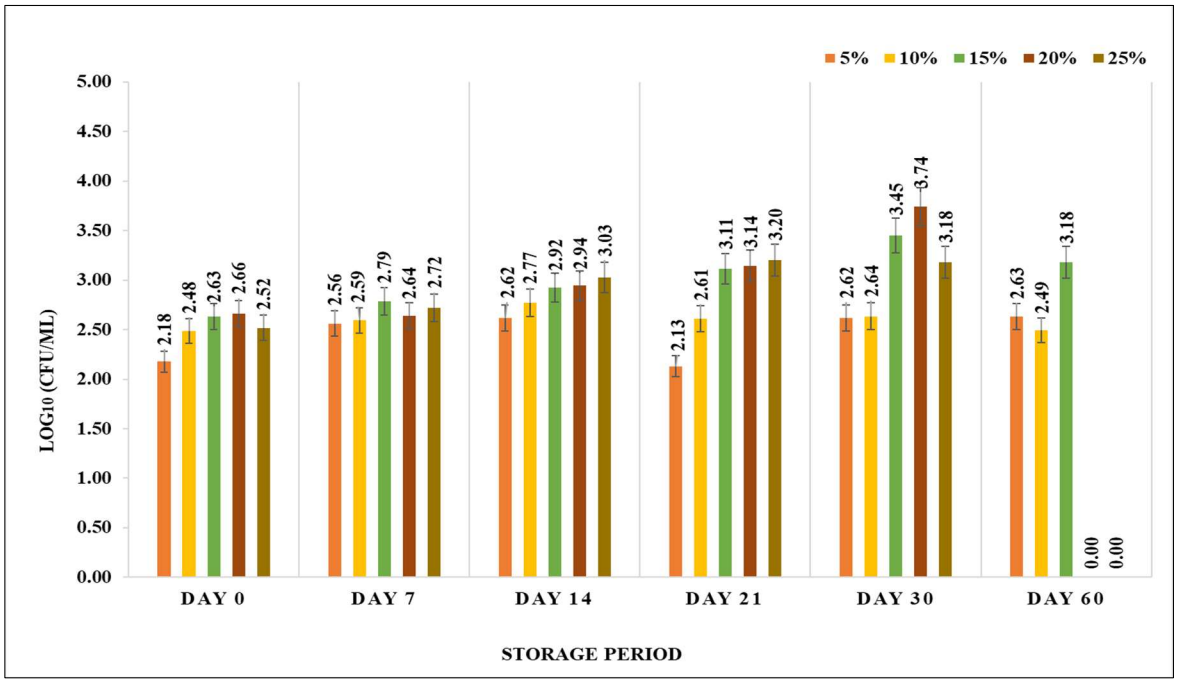


Figure 13. Survival of agar grown inoculum of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% fish protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

The inoculum of *S. enterica* grown on agar medium (2.74 Log_{10} culture) was tested at 5, 10, 15, 20, and 25% concentrations of FPH and 5±2°C for a storage period of 60 days. The comparison of inoculum prepared from broth and agar showed that the latter offers a better survival along with an increase in the number of *S. enterica* cells as compared to the former (Figures 12 and 14). The results showed recoveries of 121.29, 120.07, and 123.29%

at FPH concentrations of 5, 10, and 15 %, respectively. Although an improvement in survival was observed at 20 and 25% FPH concentrations, no growth was observed after 60 days of storage. The inoculum prepared on agar plates survived after 60 days of storage at 5 to 15% FPH concentrations. This could result from an increase in the survival capacity of the inoculum grown on agar compared to broth, as the development of resistance in *Salmonella* in response to unfavorable environmental conditions can be achieved by growing the inoculum on agar-based media (15). Several studies on the survival of *Salmonella* and *E. coli* in the presence of environmental stress have shown that inocula grown on agar are resistant to adverse conditions like drying, heat, desiccation, and cold storage, leading to an increase in the survival rate compared to broth-grown inocula (77,79,105,188). This fact can be attributed to the expression of various stress response regulons upon the transition from a liquid to a solid medium (81,189).

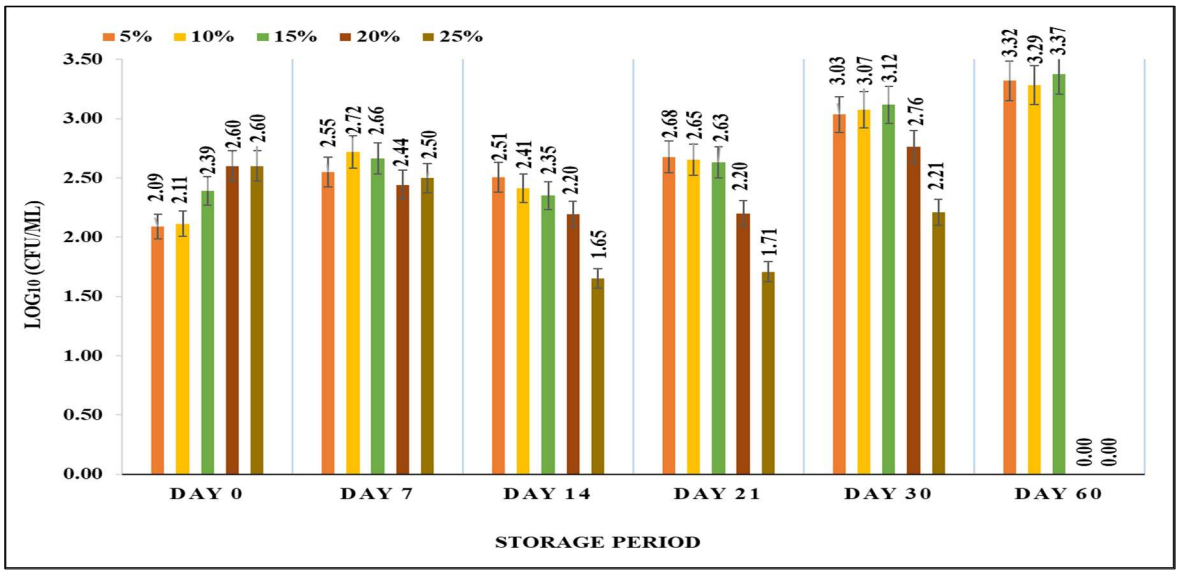


Figure 14. Survival of agar-grown inoculum of *S. enterica* subspp. Arizonae MTCC 660^{T} in the presence of 5, 10, 15, 20 & 25% fish protein hydrolysate stored at 5±2°C for 60 days. Graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, 30, and 60 days with error bars at 95% confidence interval and n=2 for each response.

Therefore, the inoculum grown on agar medium was used for further optimization studies using RSM.

6.2 Statistical optimization of protein hydrolysates through response surface methodology

To evaluate synergistic effects among the hydrolysates that could improve the survival of *E. coli* and *S. enterica* at low temperatures (5±2°C and -18±3°C), response surface methodology was used as a tool for statistical optimization Based on the analysis of individual hydrolysates, lower concentrations of these hydrolysates were selected (5% to 15%) as factors for statistical optimization. Response Surface Methodology (RSM) was employed using controllable variables (factors) at concentrations of 0, 5, and 10% for each protein hydrolysate (CH, SPH, and FPH). These concentrations were coded as -1, 0, and +1, respectively. The experimental design included three-level factor concentrations for all three protein hydrolysates, resulting in 17 experimental points or runs. These runs explored various combinations of the three protein hydrolysates. The BBD model was used because the study design met the minimum three-factor requirements of BBD, and there was more than one response that had to be monitored over an interval of 7 days for a storage period of 28 days. All the readings were taken in triplicate using blank and culture controls. The RSM study at the storage temperature of -18±3°C showed no survival from Day 7 onwards for both the target microorganisms.

6.2.1 Response surface methodology study for statistical optimization of protein hydrolysates on *E. coli* as target analyte

Based on the analysis of individual protein hydrolysates, lower concentrations of these hydrolysates were selected (0%, 5% and 10%) as factors for RSM and agar propagated culture of *E. coli* MTCC 1610^T at 2.40 Log_{10} value was used for the study. Table 7 provides details of the BBD, along with the actual recovery and predictive values for *E. coli* converted to Log_{10} values for storage temperature at 5±2°C. Statistical analysis (ANOVA) of the proposed model for the optimization of protein hydrolysates was found to be significant, as the observed values of R-squared (0.92 at 28 days) were higher, demonstrating a strong correlation between the experimental and predicted values (Table 8). The linear model was found to be satisfactory and statistically significant by the p-value

(<0.0001), non-significant lack of fit (F value 0.43), and fit statistics (adequate precision of 23.26 >4) for *E. coli* at 28 days. The relationship between the survival rate of *E. coli* and the three protein hydrolysates observed on Day 28 is shown in the equation below (7).

$$Y = 1.31 - 0.52(A) - 1.10(B) + 0.31(C) \quad (7)$$

were Y = Response Value (Survival Rate); A = Casein hydrolysate; B = Soy protein hydrolysate; C = Fish protein hydrolysate with their respective coefficient estimate factors

Through the study, variance inflation factors (VIFs) were observed to be 1.00, indicating the orthogonal nature of factors. The calculated values of deviation (actual and predicted values of activities) for *E. coli* for Day 14 (7.63%) & Day 21 (8.25%) were observed within the permitted limits (± 10%) whereas the calculated values of deviation (actual and predicted values of activities) for Day 0 (13.67%), Day 7 (38.52%), and Day 28 (22.03%) were observed outside the permitted limits (± 10%). In microbiology studies, the standard deviation is very large compared to Chemistry. The precision observed in analytical methods is very high when compared to microbiology methods. This is due to the non-homogeneous presence of microorganisms in a simple matrix like water (71,190). In almost all the research literature studied for the application of RSM in the optimization of microbial culture, the captured response is analytical in nature, like the production of metabolites and their estimation, rather than viability studies (191). According to ISO 33405:2024, which outlines procedures for preparing RM, the acceptable standard deviation limit is 0.35 Log_{10} (65). Applying this limit, if we observe a count of 100 CFU/mL, the acceptable range would be 45 CFU/mL to 224 CFU/mL. This study involves living microorganisms in liquid and refrigeration conditions. These conditions are not enough to provide a dormancy state to microorganisms, resulting in a higher standard deviation as the microorganisms can be at different stages of their growth cycle. Hence, RSM can be utilized for microbial viability studies for the count as a response, to get a brief idea about the optimization components that need to be based on other factors like repeatability and reproducibility of the response. Standard deviation is an effective tool and is required to be monitored as per ISO 33405 in the assigned value microbiological RM. The general rule of a 10% standard deviation cannot be applied to microbiological studies

involving microbial counts as a response rather than microbial metabolites as a response (192,193). The results of the viability study for one of the RSM runs are represented in Figure 15. The effect of CH, SPH & FPH on the survival of *E. coli* MTCC 1610^{T} is further elaborated by the 3-D surface contour graph (Figure 16), where the maximum response is achieved at 5% FPH in the absence of CH and SPH. Different types of protein hydrolysates have been evaluated on *E. coli* for replacement of peptones in microbiological growth media, where different protein hydrolysates from fish are the most promising as compared to commercial peptones and act as a source of nitrogen, amino acids, and vitamins (194,195). A similar result was observed in the 3-D surface contour graph (Figure 16), where FPH provided the highest response and acted as a better nutritive agent than CH and SPH.

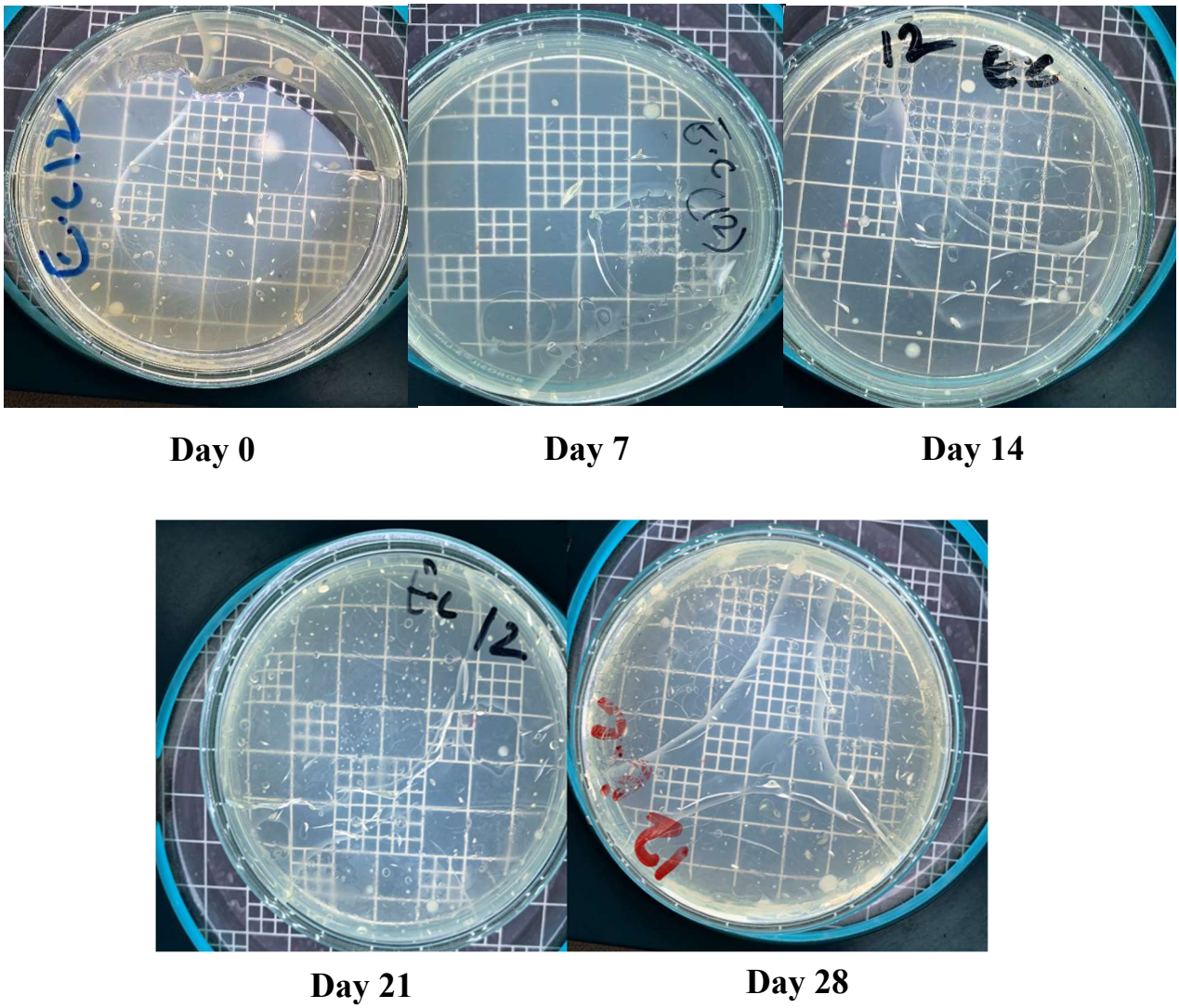


Figure 15. Photograph of viability results observed during RSM study optimization of protein hydrolysate on *E. coli* MTCC 1610^{T} at 5±2°C.

Table 7: Box Behnken design for optimization of protein hydrolysates along with actual and predicted values of *E. coli* MTCC 1610^{T} recovery value converted to Log_{10} value

	Independent variables						Response 1		Response 2		Response 3		Response 4		Response 5	
	A	B	C	A:CH	B:SPH	C:FSH	Day 0		Day 7		Day 14		Day 21		Day 28	
				(%)	(%)	(%)	Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10}(CFU/mL)	
Run	Coded levels			Actual levels			AV	PV	AV	PV	AV	PV	AV	PV	AV	PV
1	+1	+1	0	10	10	5	0.30	0.29	0.30	-0.21	0.30	0.32	0.30	0.14	0.00	-0.32
2	0	+1	+1	5	10	10	0.60	0.58	0.48	0.39	1.00	0.99	0.78	0.84	0.3	0.51
3	0	0	0	5	5	5	1.81	1.59	0.4	1.52	1.51	1.46	1.36	1.46	0.48	1.31
4	0	-1	-1	5	0	0	2.52	2.60	2.54	2.66	1.99	1.93	1.93	2.07	2.32	2.10
5	0	+1	-1	5	10	0	0.70	0.76	0.48	0.55	0.48	0.45	0.3	0.34	0.00	-0.11
6	0	-1	+1	5	0	10	2.63	2.43	2.99	2.49	2.59	2.47	2.64	2.57	2.73	2.72
7	-1	0	+1	0	5	10	1.80	1.89	1.99	2.12	2	2.13	2.08	2.16	2.37	2.14
8	0	0	0	5	5	5	1.53	1.59	1.98	1.52	1.38	1.46	1.52	1.46	1.4	1.31
9	-1	+1	0	0	10	5	1.18	1.06	0.3	1.15	1.2	1.12	1.04	1.04	0.9	0.73
10	+1	-1	0	10	0	5	2.48	2.13	1.44	1.89	1.72	1.80	1.94	1.87	2.04	1.89
11	+1	0	+1	10	5	10	0.90	1.12	0.48	0.76	1.26	1.33	1.32	1.26	1.2	1.09
12	-1	-1	0	0	0	5	2.53	2.90	3.02	3.26	2.60	2.60	2.85	2.77	2.93	2.93
13	+1	0	-1	10	5	0	1.08	1.30	0.6	0.92	0.78	0.79	0.7	0.76	0.30	0.48
14	-1	0	-1	0	5	0	2.34	2.07	2.97	2.29	1.46	1.59	1.89	1.66	1.51	1.52
15	0	0	0	5	5	5	1.48	1.59	2.03	1.52	1.71	1.46	1.28	1.46	1.18	1.31
16	0	0	0	5	5	5	1.45	1.59	2.26	1.52	1.53	1.46	1.36	1.46	1.2	1.31
17	0	0	0	5	5	5	1.76	1.59	1.62	1.52	1.34	1.46	1.45	1.46	1.34	1.31

Actual values (AV); predicted values (PV)

Table 8: Statistical analysis (ANOVA) by response surface methodology of the proposed model for survival of *E. coli* MTCC 1610^T leading to optimization of protein hydrolysates when stored at 5±2°C over a period of 28 days

Model	Day 0	Porb>F	Day 7	Porb>F	Day 14	Porb>F	Day 21	Porb>F	Day 28	Porb>F
	8.06 (Significant)	**<0.0001**	**12.66 (Significant)**	**0.0004**	**6.23 (Significant)**	**< 0.0001**	**8.14 (Significant)**	**< 0.0001**	**12.66 (Significant)**	**< 0.0001**
A	1.19	0.0002	3.73	0.0058	1.28	< 0.0001	1.62	< 0.0001	2.17	0.0002
B	6.81	< 0.0001	8.88	0.0002	4.38	< 0.0001	6.02	< 0.0001	9.72	< 0.0001
C	0.0630	0.2701	0.0528	0.7015	0.5724	< 0.0001	0.5000	< 0.0001	0.7626	0.0096
R^2	0.9289		0.7391		0.9747		0.9775		0.9216	
Adj. R^2	0.9125		0.6789		0.9689		0.9723		0.9036	
Lack of fit	Not Significant		Not Significant		Not Significant		Not Significant		Not Significant	

Probability value of Fisher's variance ratio (Prob>F), coefficient of determination (R^2)
The concentration of CH, SPH and FSH is represented by independent variables A, B and C.
The effect of variables in squared terms (A^2, B^2, C^2)
Interactive effect of the variables (AB, AC, BC)

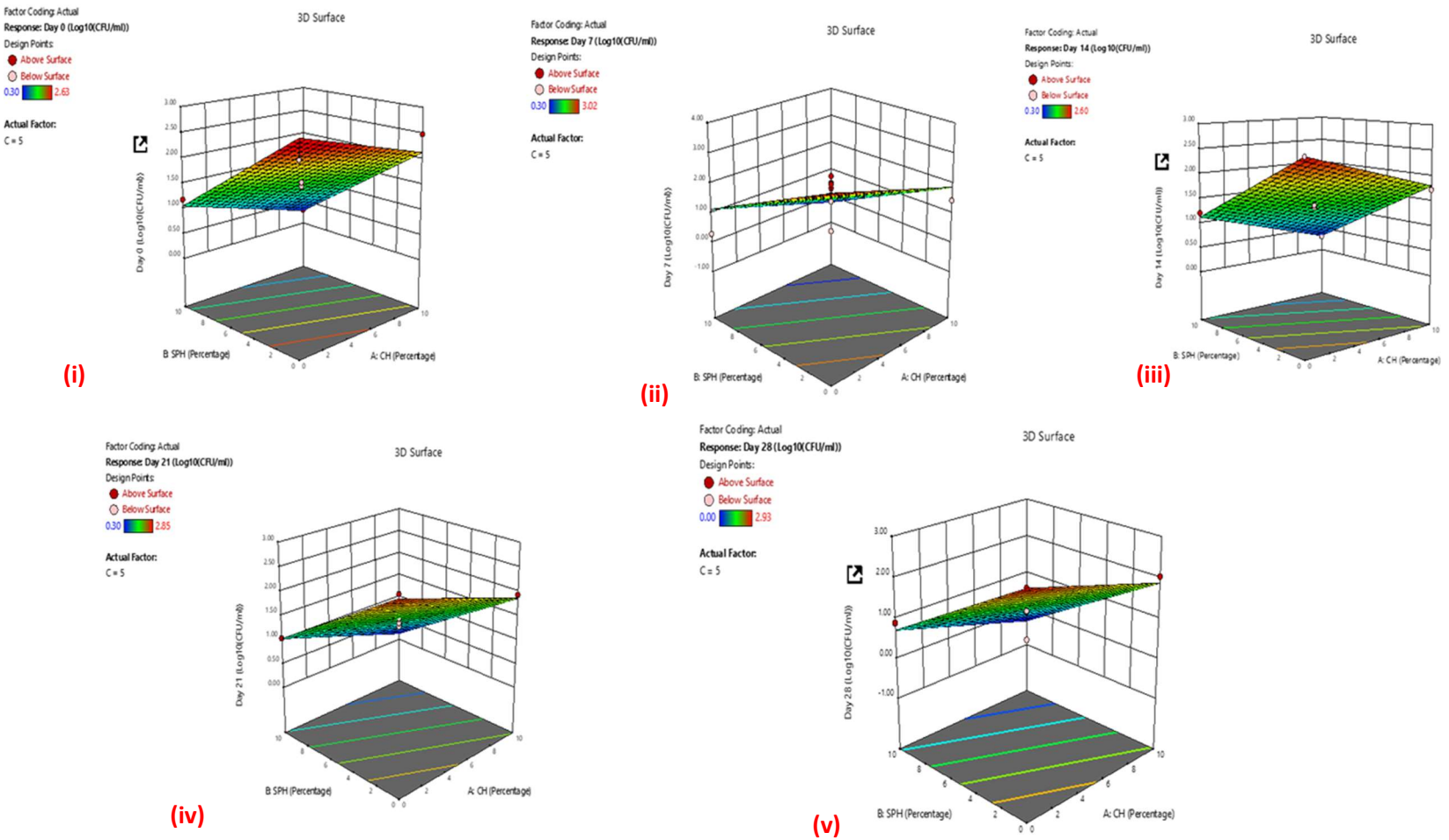


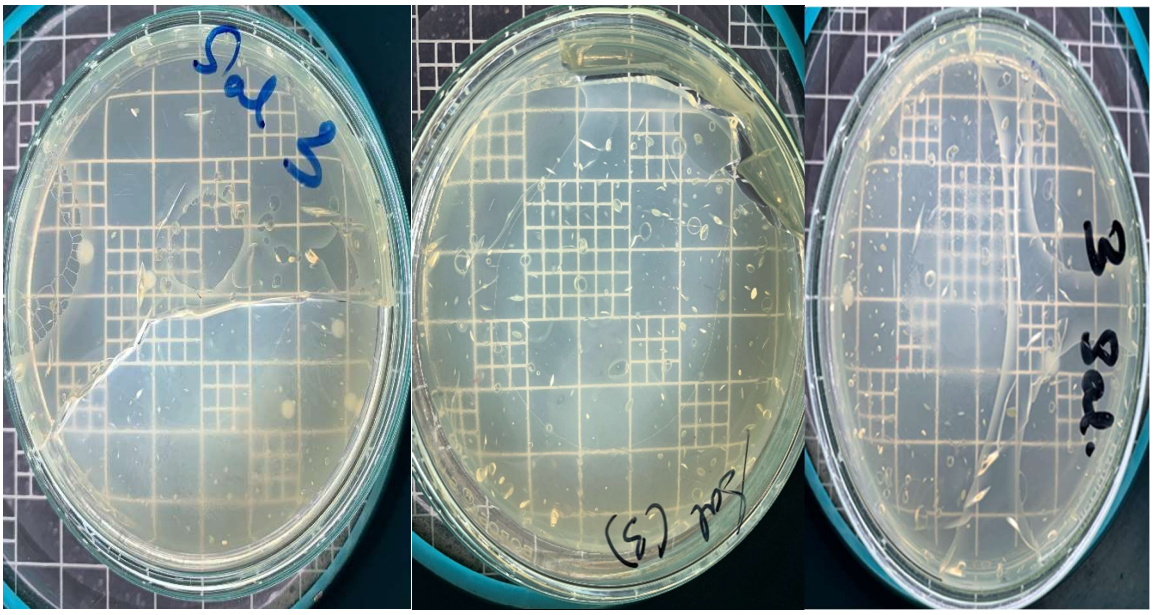
Figure 16. 3-D Contours showing the effect of casein hydrolysate (A:CH), soy protein hydrolysate (B:SPH) and fish protein hydrolysate (C:FPH) on the survival of *E. coli* MTCC 1610^{T} at 5±2°C on (i) Day 0, (ii) Day 7, (iii) Day 14, (iv) Day 21 and (v) Day 28

6.2.2 Response surface methodology study for statistical optimization of protein hydrolysates on *S. enterica* as target analyte

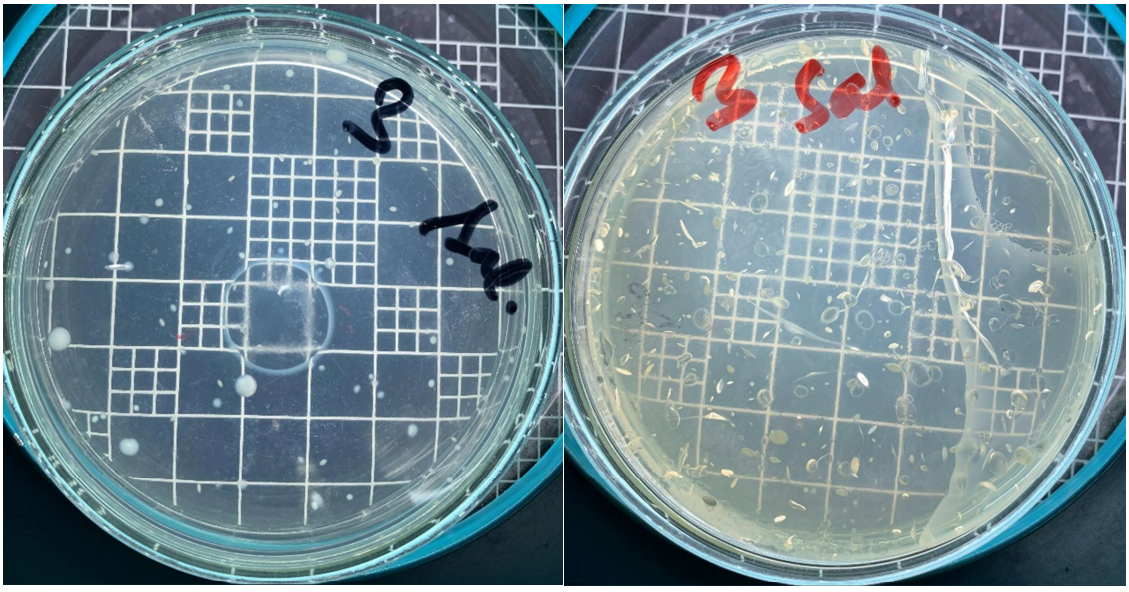
As per the outcome of individual protein hydrolysate study, lower concentrations of these hydrolysates were selected (0%, 5% and 10%) as factors for RSM study with *S. enterica* as target microorganism. Agar propagated culture of *S. enterica* MTCC 660^T at 2.38 Log_{10} culture was used as the initial inoculum for the study Table 9 provides details of the BBD, along with the actual recovery and predictive values for *S. enterica* converted to Log_{10} values for storage temperature at 5±2°C. ANOVA of the proposed model for the optimization of protein hydrolysates was found to be significant, as the model F- values were higher (291.56 at Day 0, 403.13 at Day 7, 345.50 at Day 14, 210.35 at Day 21, and 403.15 at Day 28) and the model p-values were <0.0001, which needs to be <0.05 to confirm the significance of the model (196,197). Similarly, the predicted R^2 value of 0.9799 (Day 28) closely matched the adjusted R^2 value of 0.9956 (Day 28), with a difference of less than 0.2. This result (Table 10) demonstrates a strong correlation between the experimental and predicted values (61). The coefficients of variance (CV) observed for the model were 3.50, 6.89, 4.74, 9.90, and 4.58% at 0, 7, 14, 21, and 28 days, respectively. The observed value of CV was less than 10%, indicating that the model was reproducible (198). The adequate precision determines the variation in the response relative to the target under varying noise conditions, where a value greater than 4 is desired (199,200). Specifically, the adequate precision for the model was observed to be 60.18, 68.93, 62.60, 45.46, and 70.08 for 0, 7, 14, 21, and 28 days, respectively. The overall linear model was satisfactory and highly statistically significant (model $p < 0.0001$, leading to a confidence limit of >99%) for *S. enterica*. For Days 0 and 28, the quadratic model was also satisfactory and highly statistically significant (model p-value ≤ 0.0001, leading to a confidence limit of >99%). The relationship between the survival rates of *S. enterica* and the three protein hydrolysates observed on Day 28 is shown in equation 8.

$$Y = 1.91 - 0.32(A) - 1.16(B) + 0.37(C) + 0.16(AB) - 0.13(AC) - 0.26(BC) - 0.04(A)^2 + 0.20(B)^2 + 0.11\,(C)^2 \quad (8)$$

Throughout the study, variance inflation factors (VIFs) were observed to be 1.00, indicating their orthogonal nature. Multicollinearity between the independent variables was assessed using the VIFs, and because the VIFs were observed to be 1.00, no correlation was observed between the independent variables (201). Calculated deviation values (actual and predicted values of activities) for *S. enterica* at Days 0 (3.50%), 7 (6.89%), 14 (4.74%), 21 (9.90%), and 28 (4.58%) were observed within the allowed limits (±10%), therefore, showing the model to be satisfactory and statistically significant (202,203). The signal-to-noise ratio for *S. enterica* at Days 0 (60.18), 7 (68.927), 14 (62.601), 21 (45.458), and 28 (70.083) was greater than the desired ratio (4.0), further indicating the accuracy of the proposed model (204). Photographic representation of viability study results for one of the RSM runs is represented in Figure 17. The effects of CH, SPH, and FPH on *S. enterica* survival were further elaborated by 3-D surface contour graphs (Figure 18), where the maximum survival response was achieved at 5% FPH in the absence of CH and SPH.



Day 0 Day 7 Day 14



Day 21 Day 28

Figure 17. Photograph of viability results observed during RSM study optimization of protein hydrolysate on *S. enterica* subspp. Arizonae MTCC 660^{T} at 5±2°C.

Table 9*:* Box Behnken design for optimization of protein hydrolysates along with actual and predicted values of *S. enterica* subspp. Arizonae MTCC 660^{T} recovery value converted to Log_{10} value

	Independent variables						Response 1		Response 2		Response 3		Response 4		Response 5	
Run	A	B	C	A:CH (%)	B: SPH (%)	C: FSH (%)	Day 0 Log_{10}(CFU/mL)		Day 7 Log_{10}(CFU/mL)		Day 14 Log_{10}(CFU/mL)		Day 21 Log_{10}(CFU/mL)		Day 28 Log_{10}(CFU/mL)	
	Coded levels			Actual levels			AV	PV	AV	PV	AV	PV	AV	PV	AV	PV
1	+1	0	+1	10	5	10	1.18	1.24	0.78	0.83	1.18	1.22	1.18	1.17	1.20	1.19
2	0	0	0	5	5	5	1.57	1.53	1.38	1.42	1.49	1.44	1.38	1.40	1.28	1.19
3	-1	-1	0	0	0	5	2.78	2.81	3.02	3.06	2.52	2.48	2.85	2.94	3.03	2.99
4	+1	-1	0	10	0	5	2.39	2.33	1.78	1.89	1.72	1.75	2.22	2.22	2.08	2.04
5	-1	+1	0	0	10	5	1.11	1.17	0.90	0.95	1.20	1.14	0.60	0.59	0.30	0.35
6	0	0	0	5	5	5	1.53	1.53	1.53	1.42	1.38	1.44	1.60	1.40	1.18	1.19
7	+1	0	-1	10	5	0	1.18	1.21	0.78	0.85	0.90	0.93	0.90	0.92	0.70	0.72
8	0	0	0	5	5	5	1.49	1.53	1.41	1.42	1.49	1.44	1.20	1.40	1.15	1.19
9	0	0	0	5	5	5	1.54	1.53	1.40	1.42	1.41	1.44	1.20	1.40	1.18	1.19
10	0	+1	-1	5	10	0	0.70	0.70	0.30	0.38	0.70	0.63	0.00	0.11	0.30	0.25
11	0	0	0	5	5	5	1.51	1.53	1.36	1.42	1.36	1.44	1.51	1.40	1.18	1.19
12	0	-1	-1	5	0	0	2.52	2.55	2.54	2.48	1.99	1.97	2.63	2.45	2.01	2.04
13	-1	0	+1	0	5	10	1.80	1.77	1.99	2.00	1.93	1.96	2.08	1.89	2.09	2.07
14	0	-1	+1	5	0	10	2.43	2.43	2.59	2.47	2.36	2.26	2.60	2.70	3.24	3.30
15	-1	0	-1	0	5	0	2.10	2.04	2.11	2.02	1.57	1.67	1.63	1.64	1.08	1.09
16	0	+1	+1	5	10	10	0.60	0.57	0.30	0.36	0.85	0.9179	0.30	0.36	0.48	0.45
17	+1	+1	0	10	10	5	0.30	0.27	0.00	-0.22	0.48	0.41	0.00	-0.13	0.00	0.04

Actual values (AV); predicted values (PV)

Table 10*:* Statistical analysis (ANOVA) by response surface methodology of the proposed model for survival of *S. enterica* subspp. Arizonae MTCC 660^{T} leading to optimization of protein hydrolysates when stored at 5±2°C over a period of 28 days

	Day 0	Porb>F	Day 7	Porb>F	Day 14	Porb>F	Day 21	Porb>F	Day 28	Porb>F
Model	**7.93**	**<0.0001**	**11.62**	**< 0.0001**	**4.84**	**< 0.0001**	**2.19**	**< 0.0001**	**13.31**	**< 0.0001**
	(Significant)		**(Significant)**		**(Significant)**		**(Significant)**		**(Significant)**	
A	0.9384	< 0.0001	2.74	< 0.0001	1.08	< 0.0001	1.02	< 0.0001	0.7938	< 0.0001
B	6.86	< 0.0001	8.88	< 0.0001	3.59	< 0.0001	11.04	< 0.0001	10.76	< 0.0001
C	0.0300	0.0161	0.0006	0.8046	0.1682	< 0.0001	0.1250	0.0245	1.07	< 0.0001
A^2	0.0150	0.0610	-	-	-	-	-	-	0.0070	0.2098
B^2	0.0138	0.0700	-	-	-	-	-	-	0.1672	0.0003
C^2	0.0022	0.4239	-	-	-	-	-	-	0.0550	0.0061
AB	0.0441	0.0065	-	-	-	-	-	-	0.1056	0.0010
AC	0.0225	0.0294	-	-	-	-	-	-	0.0650	0.0040
BC	0.0000	0.9301	-	-	-	-	-	-	0.2756	< 0.0001
R^2	0.9973		0.9894		0.9876		0.9798		0.9981	
Adj. R^2	0.9939		0.9869		0.9848		0.9752		0.9956	

Lack of fit	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant

Probability value of Fisher's variance ratio (Prob>F), coefficient of determination (R^2)

The concentration of CH, SPH and FSH is represented by independent variables A, B and C.

The effect of variables in squared terms (A^2, B^2, C^2)

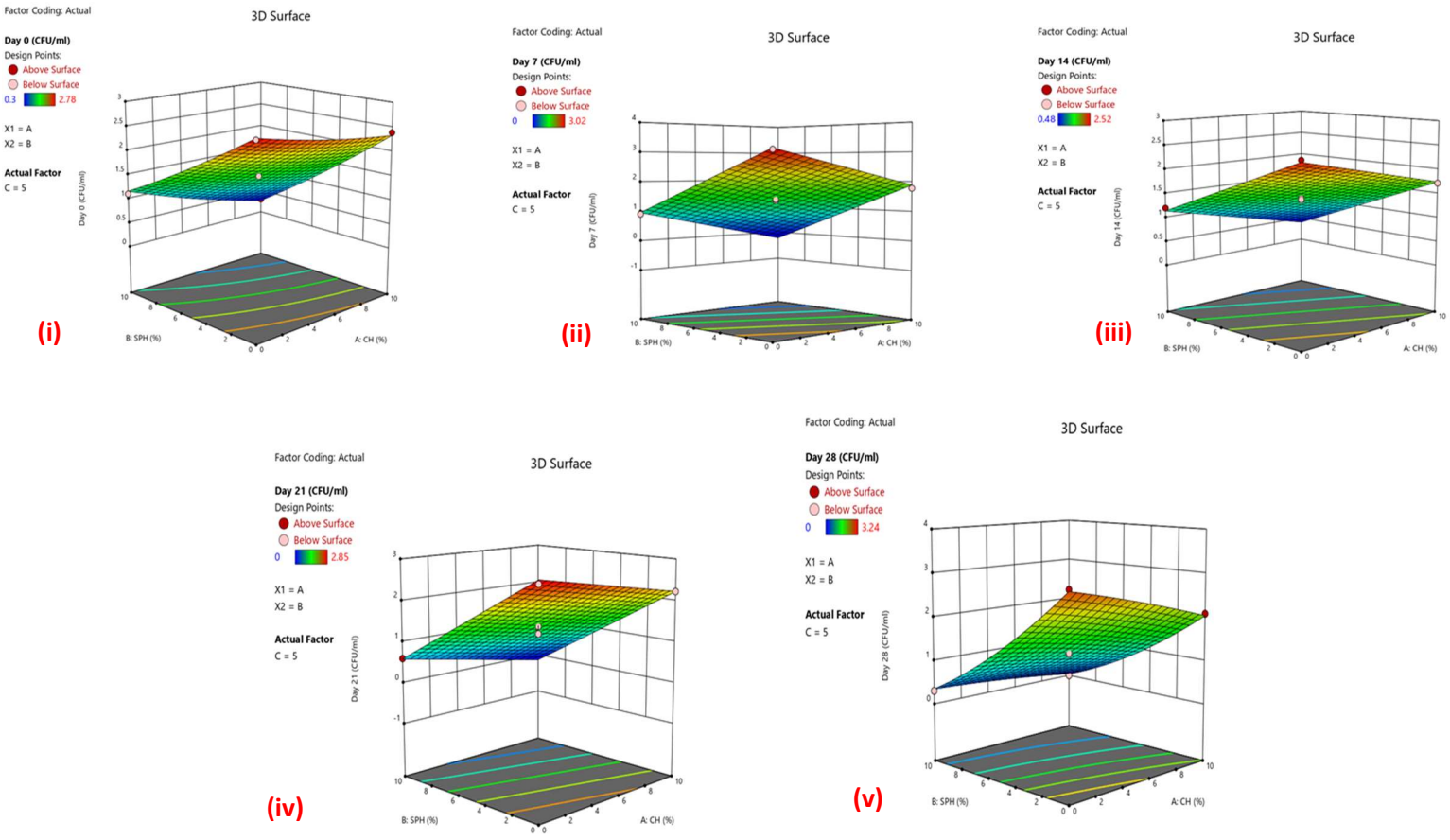


Figure 18. 3-D Contours showing the effect of casein hydrolysate (A:CH), soy protein hydrolysate (B:SPH) and fish protein hydrolysate (C:FPH) on the survival of *S. enterica* MTCC 660^{T} at 5±2°C on (i) Day 0, (ii) Day 7, (iii) Day 14, (iv) Day 21 and (v) Day 28

6.3 Effect of protective agents on the survival of *E. coli* and *S. enterica*

Protective agents were individually studied using 2.88 Log_{10} culture of *E. coli* and 2.67 Log_{10} culture of *S. enterica,* which was prepared using serial dilution of overnight grown culture in trypticase soya broth (TSB) (59,143). Among protectives, glucose, glycerol, and trehalose were individually studied for their effectiveness in supporting the viability of *S. enterica* at 5, 10, 15, 20, and 25% whereas BSA was studied at 5 and 10% concentration. For the individual study of protective agents, two different temperatures of 5 ± 2°C and -18 ± 3°C were used for storage for 30 days. The bacterial viability was assessed at regular intervals of 0, 7, 14, 21, and 30 days using trypticase soya agar (TSA) in triplicate. Based on the results of individual studies on protective agents, an optimization study using RSM was conducted to identify the best suitable combinations and concentrations of protective agents (glucose, glycerol, trehalose & BSA). Response from the RSM study was tested for the quadratic model utilizing BBD methodology. At a later stage, the encapsulation step was introduced to formulate the quantitative RM of *E. coli* and *S. enterica,* which was checked for homogeneity and stability.

6.3.1 Effect of glucose on the survival of *E. coli* and *S. enterica*

Glucose at 5, 10, and 15% concentrations was effective in the survival of *E. coli* at 5±2°C and showed a recovery of 107.73, 111.47, and 99.18%, respectively, at 30 days. Whereas higher glucose concentrations (20 and 25%) showed loss of viability on day 14, though at 25% concentration, there was a sudden recovery of 48.57% observed on day 30 (Figure 19). Under conditions of environmental stress of starvation and low temperature, the presence of glucose and other sugars results in the triggering of a small or large group of genes in *E. coli* to overcome such conditions (205). As a response to such conditions, there is an effect on cell physiology, an increase in the number of injured cells, and doubling of the generation time, which can lead to results of sudden recovery as the injured cells take time for recovery and growth (206,207). At -18 ± 3°C, glucose was able to support the survival of *E. coli* only till day 7, and no survival was observed at the 30th day of storage (Figure 20).

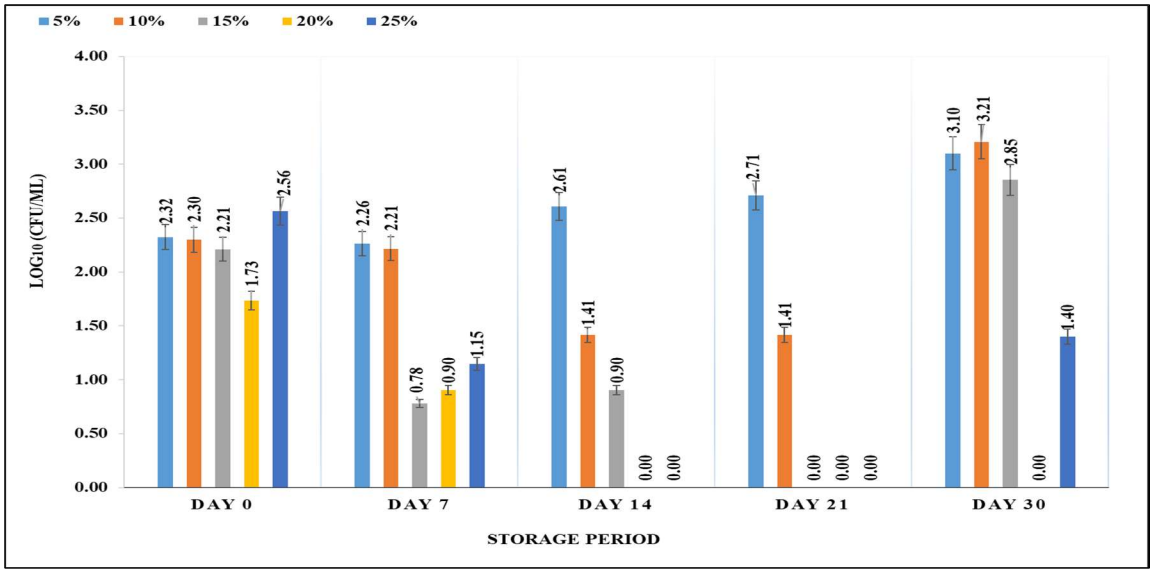


Figure 19. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25 % glucose stored at 5±2°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

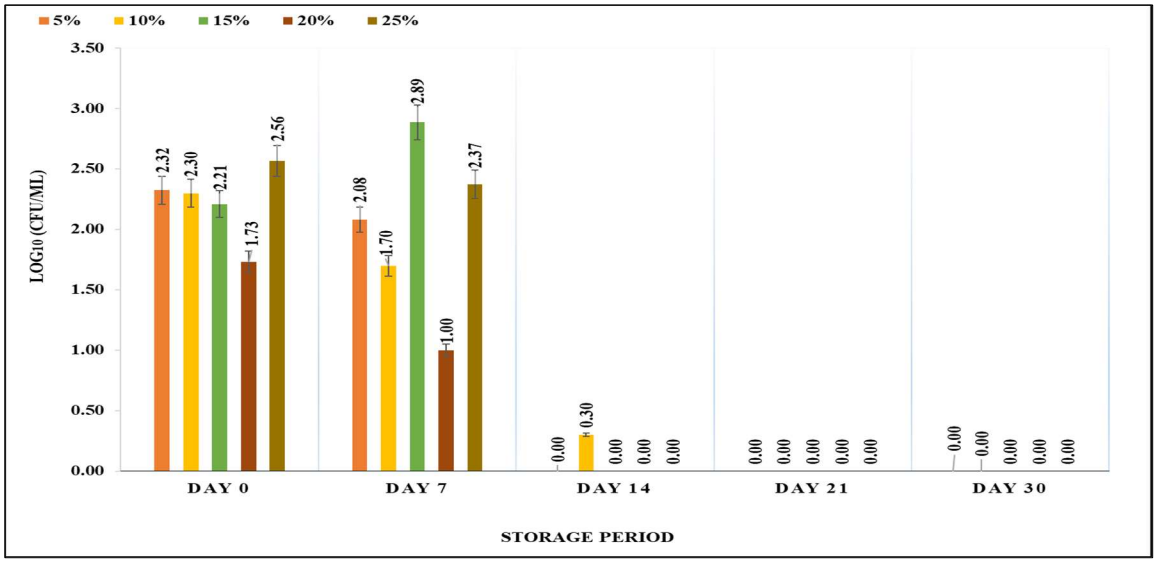


Figure 20. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% glucose stored at -18±3°C for 30 days. The graph contains viability results obtained

at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

Glucose concentrations of 5, 10, and 15% at refrigeration temperature (5±2°C) were found effective in supporting the survival of *S. enterica*, showing recovery rates of 58.72, 59.16, and 63.94%, respectively, at Day 30. However, glucose at concentrations of 20 and 25% was unable to support the viability of *S. enterica* after day 7 of storage at refrigeration temperature (Figure 21). Furthermore, all concentrations of glucose (ranging from 5 to 25%) were unable to support the survival of *S. enterica* at -18 ± 3°C, as no growth was observed after 7 days of storage (Figure 22). Freezing conditions (-20°C) result in damage to *Salmonella* cells, which leads to a reduction in the initial inoculum, and the killing effect is increased in the presence of high moisture content. The presence of sugar can reduce the killing effect, provided the relative humidity is lower than the moisture content of monolayer formation (208,209).

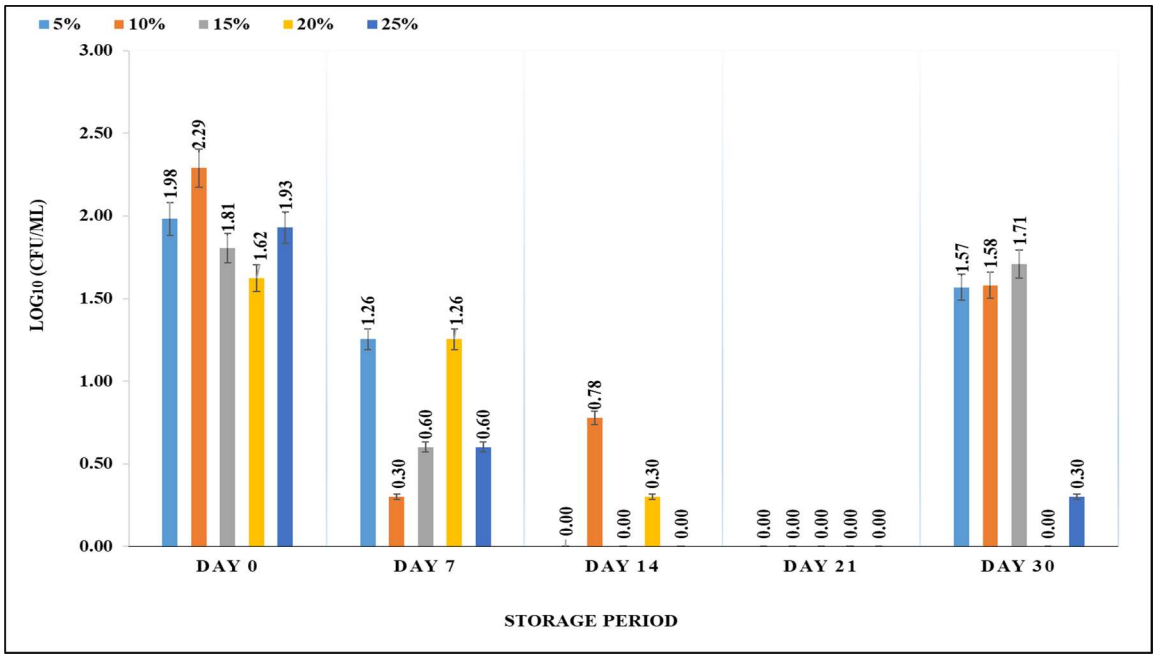


Figure 21. Survival of *S. enterica* subspp. Arizonae MTCC 660^T in the presence of 5, 10, 15, 20 & 25 % glucose stored at 5±2°C for 30 days. The graph contains viability

results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

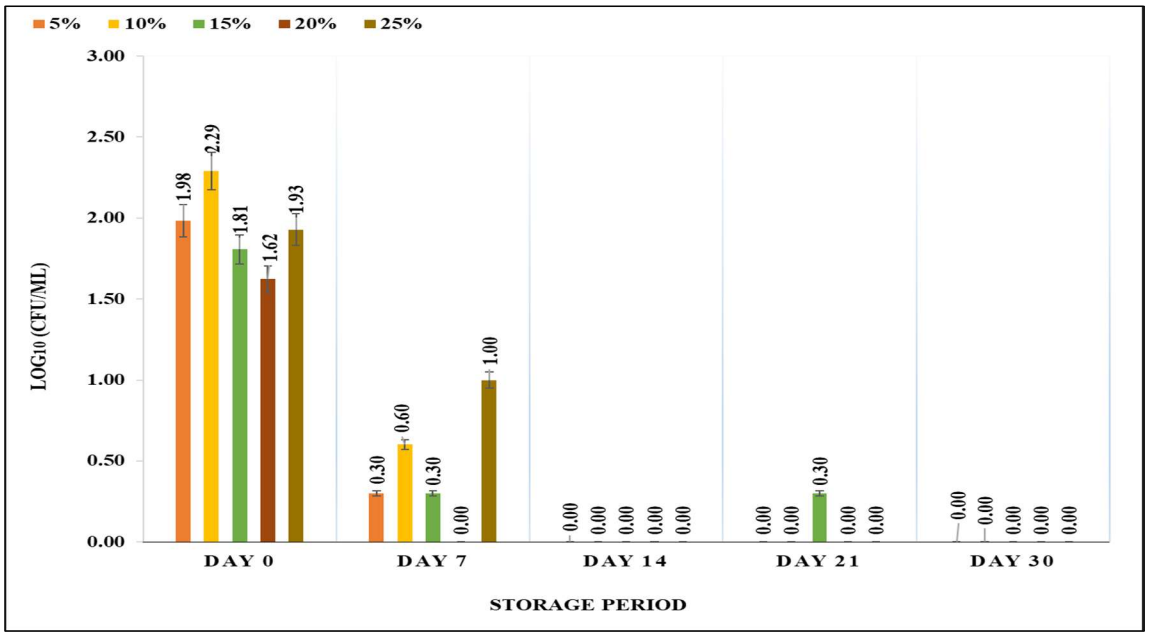


Figure 22. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in the presence of 5, 10, 15, 20 & 25% glucose stored at -18±3°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

6.3.2 Effect of glycerol on the survival of *E. coli* and *S. enterica*

Glycerol at 5% concentration supported the viability of *E. coli* by 55.67% at 5±2°C for 30 days (Figure 23), whereas all the concentrations of glycerol (5% - 20%) were observed to support the survival of *E. coli* at -18 ± 3°C (Figure 24). Glycerol concentrations of 5, 10, 15, and 20% at -18 ± 3°C showed 62.28, 43.62, 50.28, and 24.29% recovery of the target analyte, respectively. At freezing conditions, glycerol provides the most consistent protection to *E. coli* (210). Glycerol under freezing conditions can reduce cell wall and membrane damage of *E. coli* cells (211).

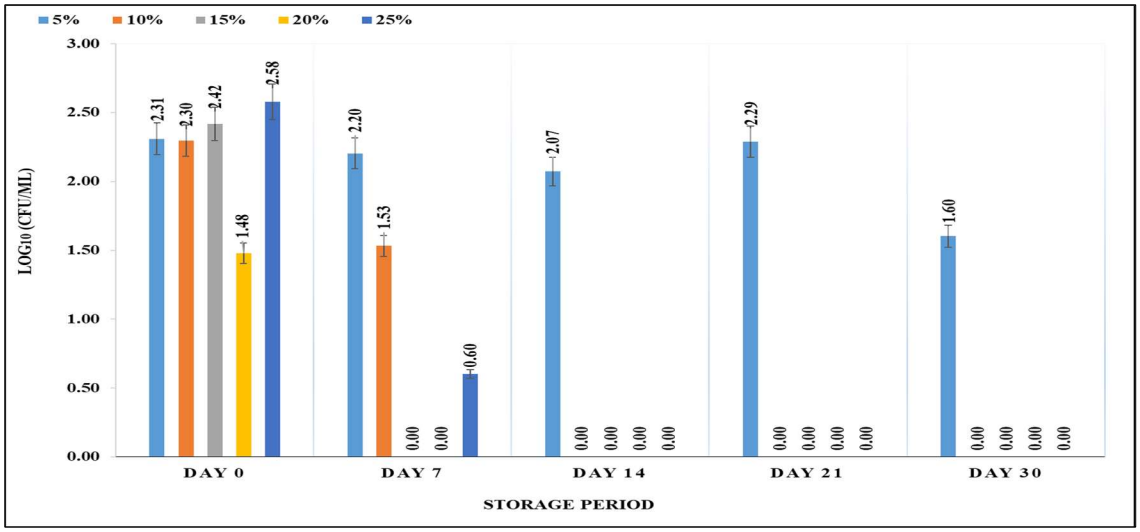


Figure 23. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% glycerol stored at 5±2°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

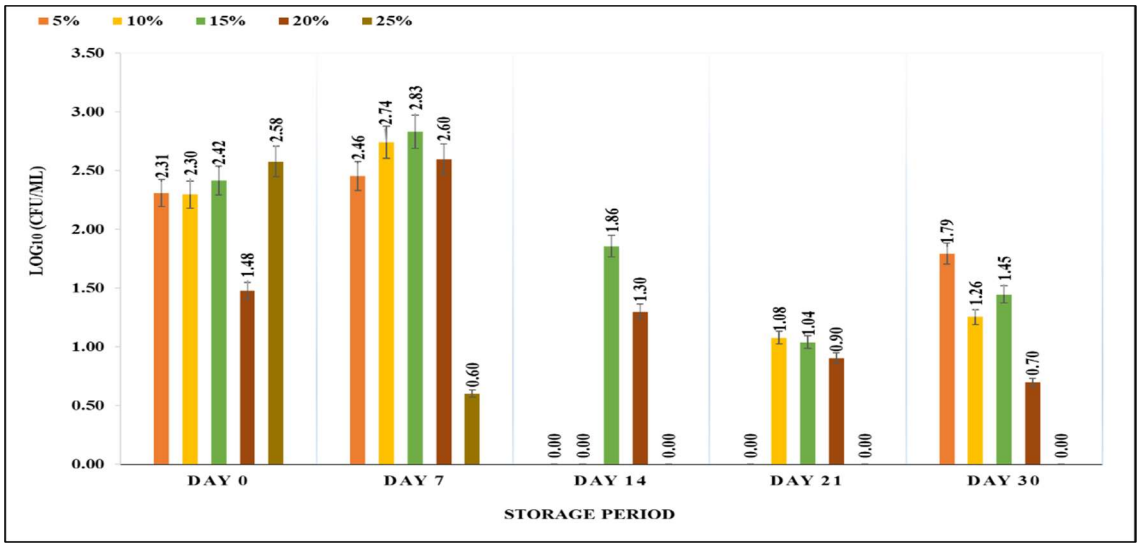


Figure 24. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% glycerol stored at -18±3°C for 30 days. The graph contains viability results obtained

at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

Glycerol, at concentrations ranging from 5 to 25% did not support the growth of *S. enterica* at 5 ± 2°C after 14 days of storage (Figure 25). However, 15 and 20% glycerol at -18 ± 3°C after 30 days demonstrated 17.86 and 67.64% viability of *S. enterica*, respectively (Figure 26). Growth of *Salmonella* in presence of lower water activity and humectants like glycerol, sodium chloride and sucrose can result in formation of filaments which is the result of osmotic stress (91,119). Presence of glycerol do not enhance recovery of injured *S. anatum* cells (212). In preservation study of 7 Log_{10} culture of *S. enterica* involving preservation media containing 10 – 20% glycerol at 4°C, -20°C and -80°C, only -80°C was effective for long term storage. There was complete loss of viability after one week for *S. enterica* at 4°C and loss of 3 to 4.7 Log_{10} at -20°C in media with 20% glycerol (213).

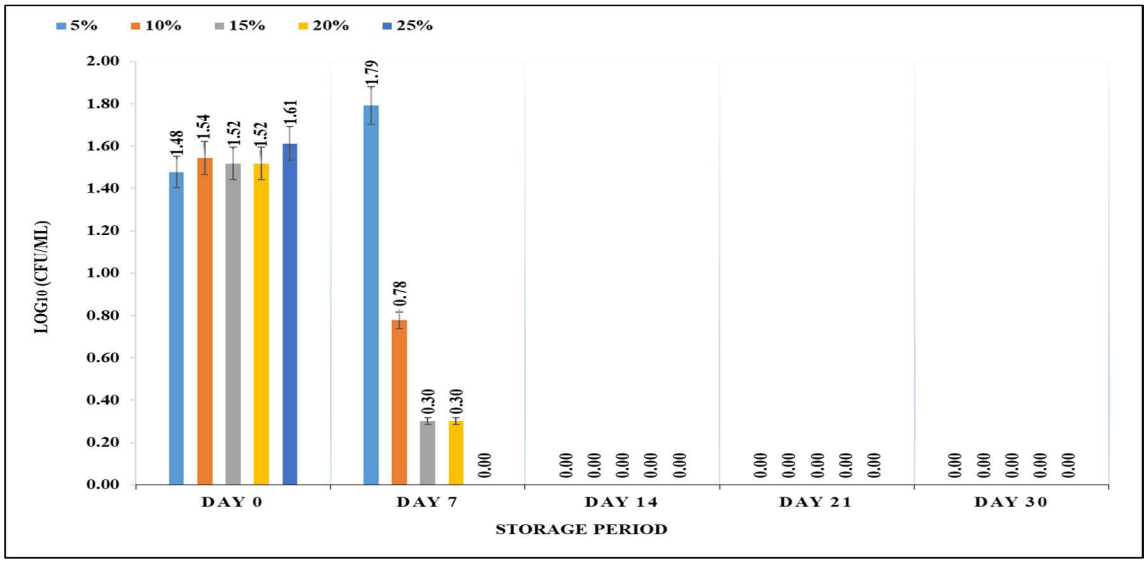


Figure 25. Survival of *S. enterica* subspp. Arizonae MTCC 660^T in the presence of 5, 10, 15, 20 & 25 % glycerol stored at 5±2°C for 30 days. The graph contains viability

results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

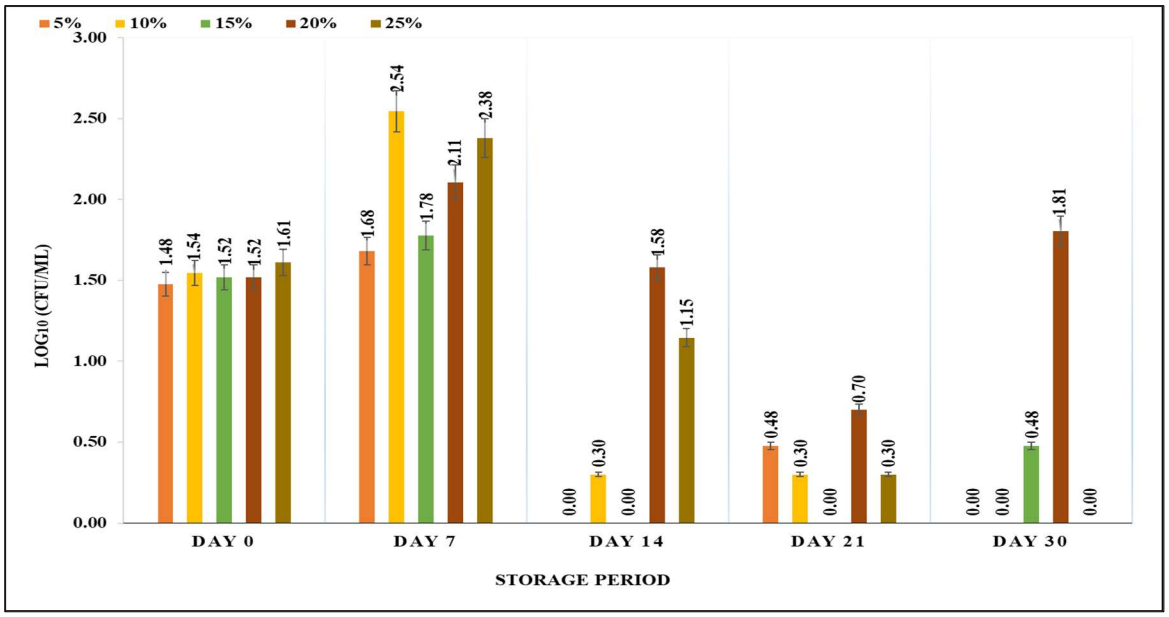


Figure 26. Survival of *S. enterica* subspp. Arizonae MTCC 660^T in the presence of 5, 10, 15, 20 & 25% glycerol stored at -18±3°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

6.3.3 Effect of trehalose on the survival of *E. coli* and *S. enterica*

All the concentrations of trehalose (5 – 25%) were found to be effective in maintaining the viability of *E. coli* MTCC 1610^T at 5±2°C. Trehalose at 5, 10, and 15% showed recovery of more than 100%, whereas 20 and 25% concentrations of trehalose showed 87.51 and 91.51% recovery at 5±2°C, respectively, at 30 days of storage (Figure 27). Trehalose was ineffective in supporting the survival of *E. coli* at -18 ± 3°C till 30 days. After 14 days of storage at -18 ± 3°C, 20 and 25% concentrations of trehalose showed 34.72 and 39.80% recovery of *E. coli,* respectively, which was reduced to no viability at 21 days of storage (Figure 28).

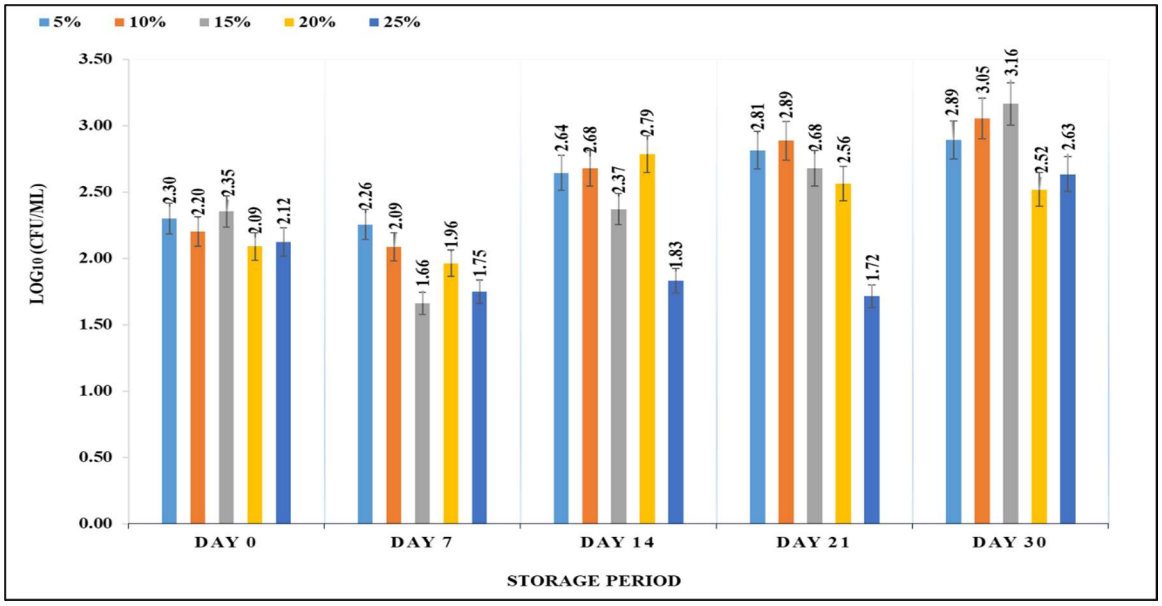


Figure 27. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% trehalose stored at 5±2°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

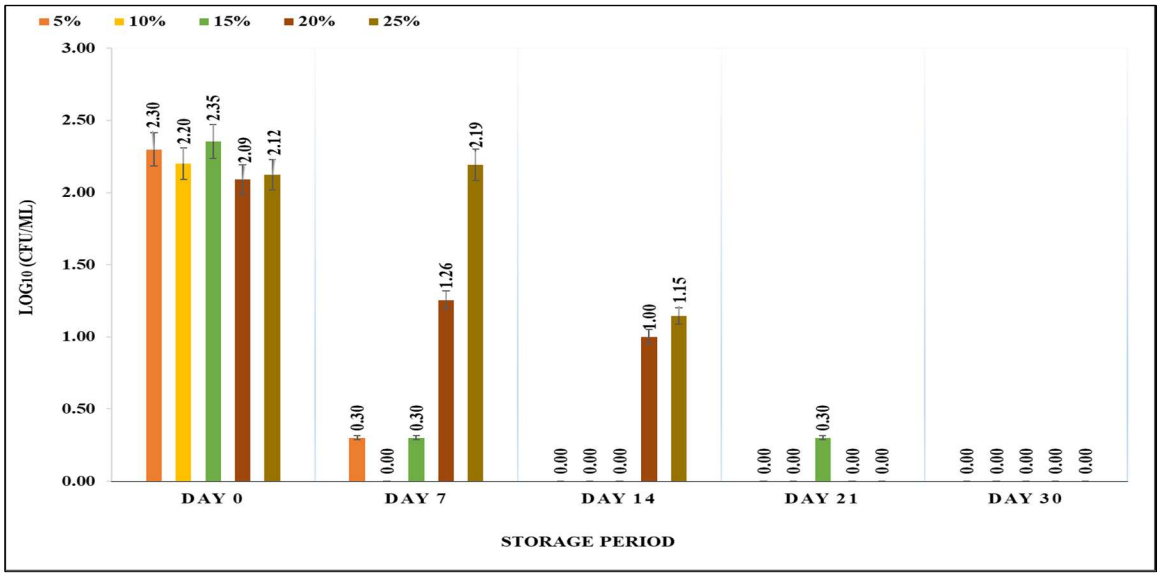


Figure 28. Survival of *E. coli* MTCC 1610^{T} in the presence of 5, 10, 15, 20 & 25% trehalose stored at -18±3°C for 30 days. The graph contains viability results obtained

at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

Only a 5% concentration of trehalose resulted in a 50.99% survival of the initially added inoculum of *S. enterica* at 5±2°C after 30 days (Figure 29). Higher concentrations of trehalose (15–25%) supported the survival of *S. enterica,* resulting in survival of 51.69%, 0.11% and 33.82% respectively at 15%, 20% and 25% of trehalose, until Day 14 under freezing conditions (-18 ± 3°C). However, beyond this point, a significant decline occurred, resulting in a survival rate of less than 11% of the initially added inoculum at -18 ± 3°C (Figure 30). Trehalose synthesis gene (*otsA* and *otsB)* in *S. enterica* serovar Typhimurium is required for environmental survival as moderate osmolality can support in development of heat resistance and support the growth of *S. enterica* at a higher limit of the growth temperature by maintaining turgor and protein stabilization (55,214) suggesting the role of trehalose in the survival of *Salmonella* at high temperatures rather than lower temperatures.

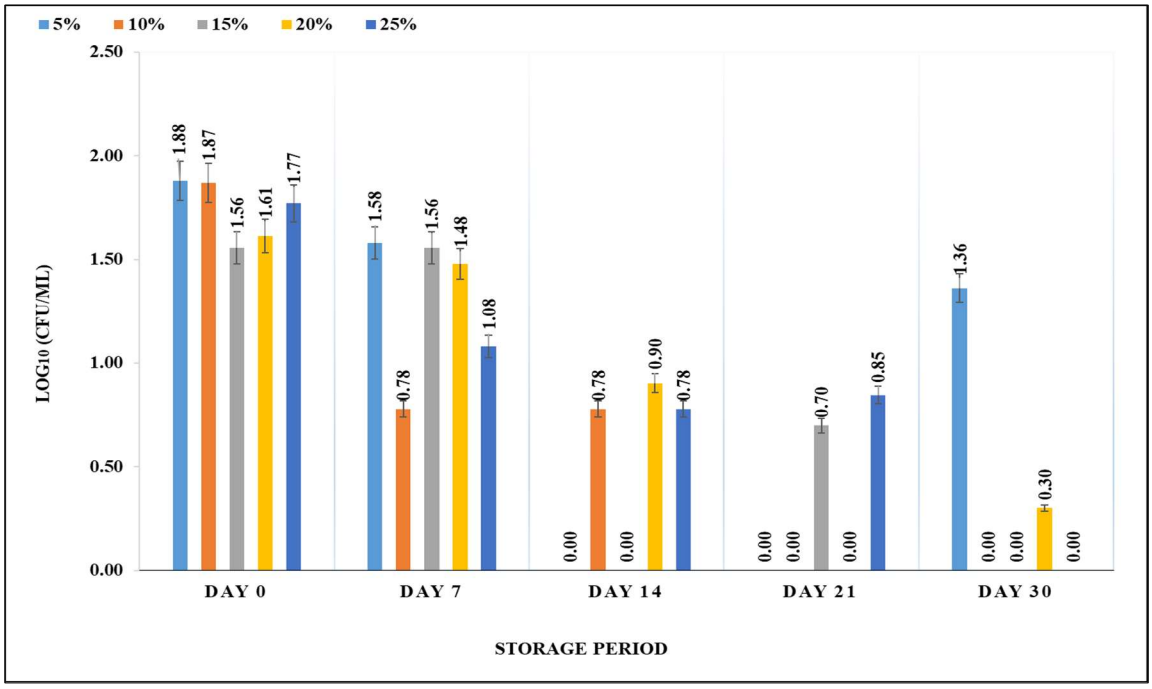


Figure 29. Survival of *S. enterica* subspp. Arizonae MTCC 660^T in the presence of 5, 10, 15, 20 & 25 % trehalose stored at 5±2°C for 30 days. The graph contains viability

results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

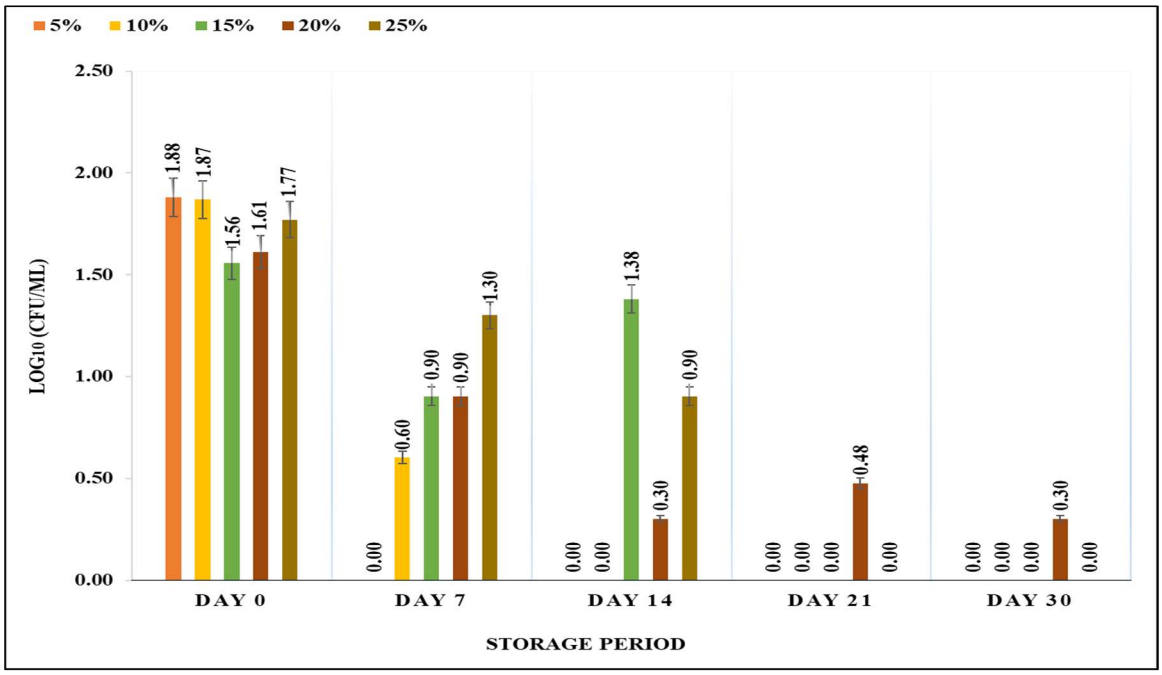


Figure 30. Survival of *S. enterica* subspp. Arizonae MTCC 660^T in the presence of 5, 10, 15, 20 & 25 % trehalose stored at -18±3°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

6.3.4 Effect of bovine serum albumin on the survival of *E. coli* and *S. enterica*

At a 5% concentration, BSA resulted in 93.17% recovery of *E. coli* at 5±2°C after 30 days of storage (Figure 31), however, it failed to provide cryoprotection at -18 ± 3°C. Only 10.46% and 27.04% recovery rates were observed for 5% and 10% BSA concentrations, respectively, after a 14-day storage period. Remarkably, no recovery was observed for both the concentrations of BSA at -18 ± 3°C on 30 days of storage (Figure 32). *Escherichia* spp. (5 Log_{10}) when subjected to overnight freeze drying at -70°C and rehydration post

lyophilization and storage at 4°C for 3 months in presence of 10% BSA showed no recovery (215).

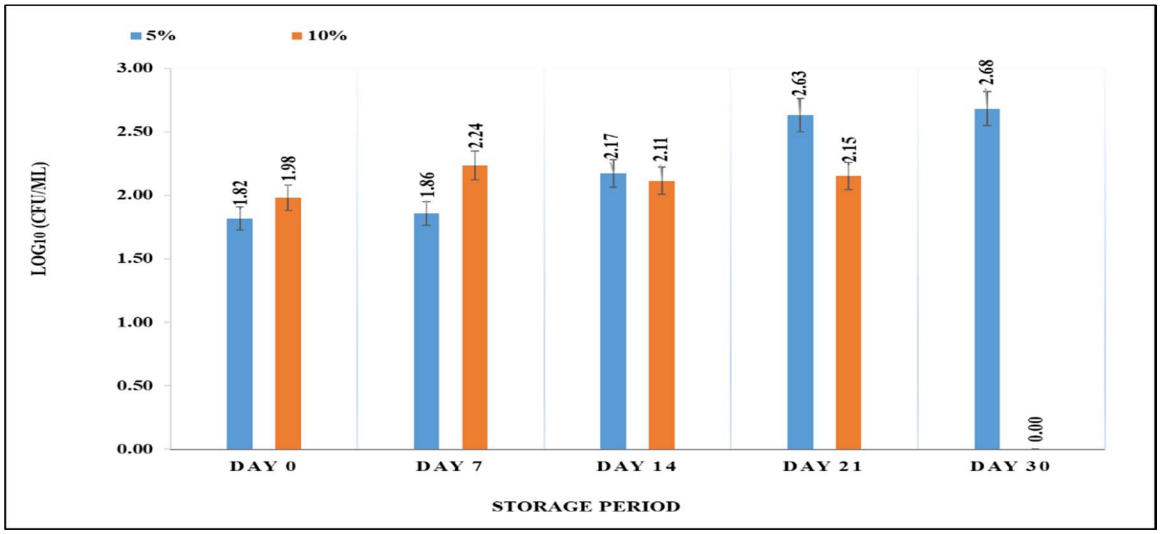


Figure 31. Survival of *E. coli* MTCC 1610^{T} in the presence of 5 & 10% BSA stored at 5±2°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

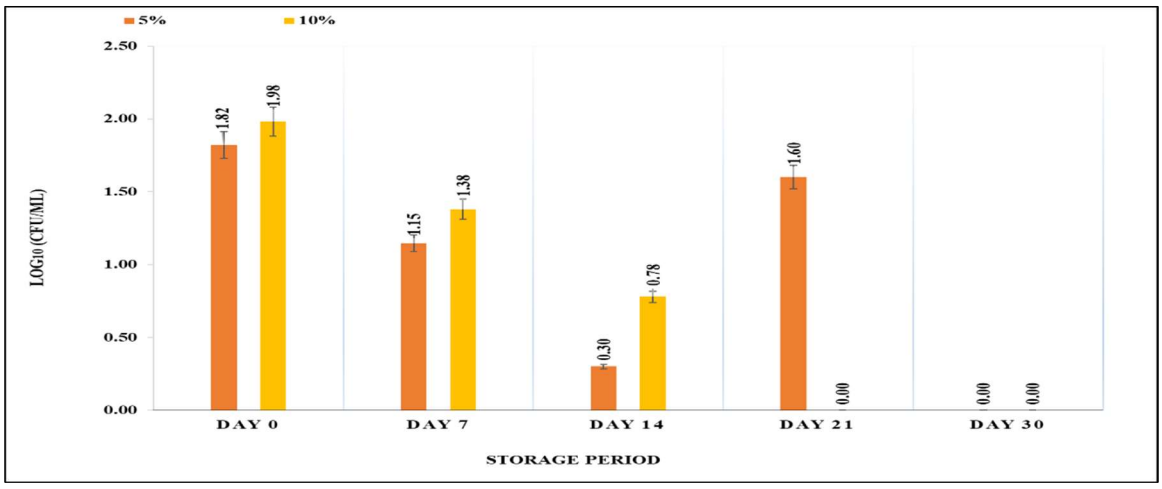


Figure 32. Survival of *E. coli* MTCC 1610^{T} in the presence of 5 & 10 % BSA stored at -18±3°C for 30 days. The graph contains viability results obtained at fixed intervals

of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

At a 10% concentration, BSA significantly improved the viability of *S. enterica* (85.84%) during refrigeration after 21 days of storage, in contrast to the 5% BSA concentration (11.27%) (Figure 33). However, no survival was observed on the 30th day for both concentrations. Parallelly, both 5% and 10% concentrations of BSA were unable to maintain the viability of *S. enterica* at -18±3°C after 14 days (Figure 34). Although we were unable to find a specific study involving the effect of BSA on the survival of *Salmonella* at lower temperatures, a similar study associated with *E. coli* suggests that BSA can impart virulence and antibiotic resistance rather than cryoprotection (216,217).

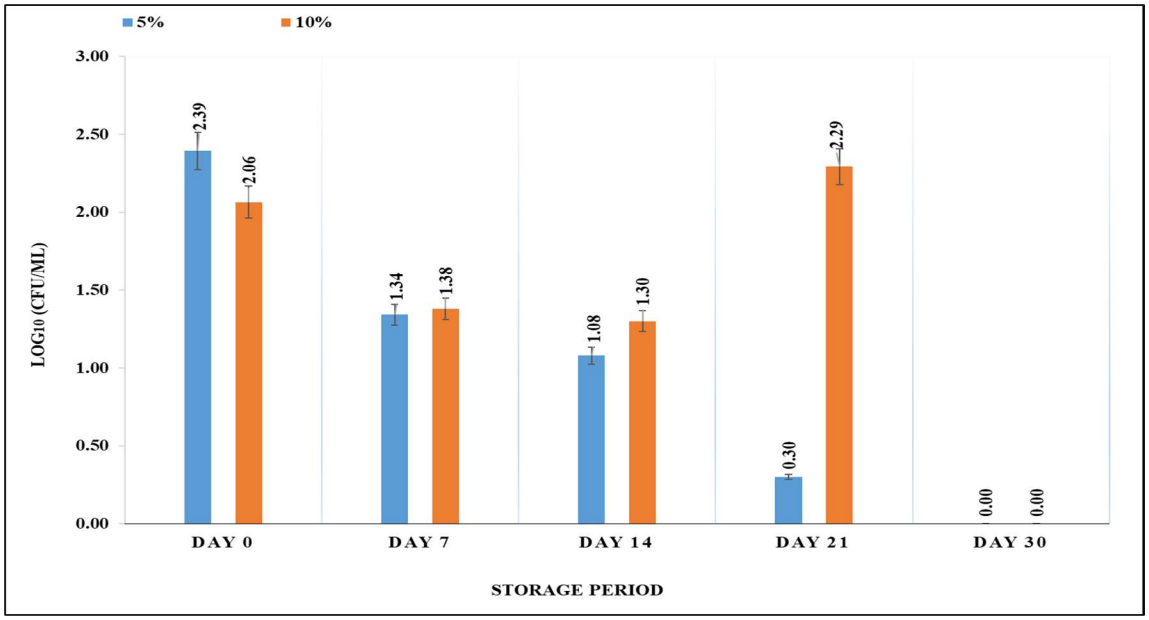


Figure 33. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in the presence of 5 & 10% BSA stored at 5±2°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

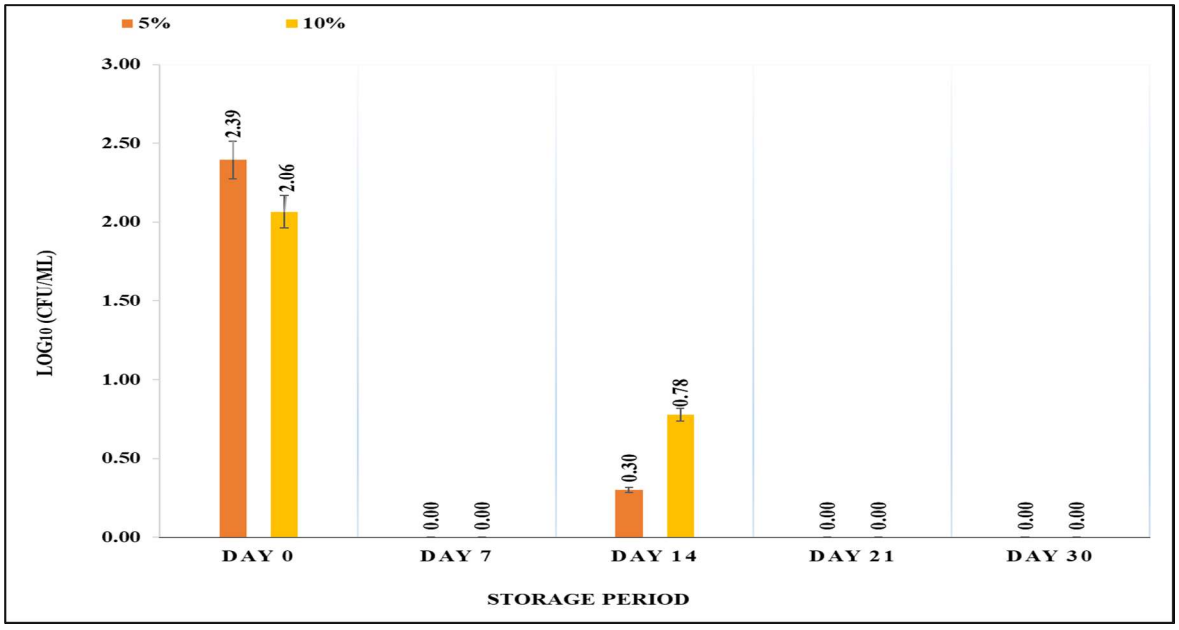


Figure 34. Survival of *S. enterica* subspp. Arizonae MTCC 660^{T} in the presence of 5 & 10 % BSA stored at -18±3°C for 30 days. The graph contains viability results obtained at fixed intervals of 0, 7, 14, 21, and 30 days with error bars at 95% confidence interval and n=2 for each response.

Replacement of cellular content with cryoprotectants results in imparting protection for microbial cell survival. Cryoprotectants are traditionally classified based on the rate of penetration into the microbial cells. The permeability of cryoprotectants is dependent on the temperature and cell type. Glycerol, polysaccharide, albumin, protein, gelatin, etc., penetrate slowly, hence they induce cryoprotection at concentrations ranging from 10 – 40%. (33,38). In this study, except for glycerol, none of the studied cryoprotectants were able to impart cryoprotection at -18 ± 3°C, which may be due to the ice crystal formation leading to the early death of microbial cells before the additives could diffuse and impart cryoprotection (218,219). Glycerol can penetrate the cell wall and cell membrane, whereas mono- and disaccharides, amino acids, and low molecular weight solvents can penetrate the cell wall only (33). Glucose at 1 – 18% concentration with a median of 4%, trehalose at 5 – 19% with a median of 10%, and serum albumin at 0.1– 4% have been used as cryoprotectants for viruses, bacteria, and yeast (220,221). The mixture of proteins and

sugars result in the formation of most effective cryoprotectants (222). Composite cryoprotectants have shown better survival rates of microbial cells by reducing cell wall and cell membrane damage, maintaining normal morphology and cell membrane integrity while improving enzyme activity during freeze drying state (223). Hence to understand the impact of all the cryoprotectants used in this study, RSM tool was applied to identify the ideal combination for the long-term survival of *E. coli* and *S. enterica*. We selected glucose, trehalose, and glycerol concentrations ranging from 5 to 15%, along with a 5% BSA concentration, for optimization using RSM.

6.4 Statistical optimization of protective agents through response surface methodology

An optimization study using RSM was conducted to identify the best suitable concentrations of protective agents (glucose, glycerol, trehalose & BSA). Responses from the RSM study were tested for the quadratic model utilizing BBD. Refrigeration and freezing temperatures were used for storage and after regular intervals, the viability was assessed using trypticase soya agar (TSA) in triplicate. In the RSM study of protectants, the selected concentrations for glucose, trehalose, and glycerol were 5, 10, and 15%, while for BSA, the selected concentrations were 0, 2.5, and 5%. The BBD model was employed as the study involved more than three factors. Multiple responses were monitored over a 28-day storage period, with measurements taken at 7-day intervals. The experimental setup included three concentration levels for glucose, trehalose, and glycerol (5%, 10%, and 15%), as well as three concentration levels for BSA (0%, 2.5%, and 5%). This design resulted in 29 experimental points for each storage temperature (5±2°C and -18±3°C). All the readings were taken in triplicate with blank and culture controls. However, the RSM study for protectants conducted at a storage temperature of -18±3°C showed no viability from Day 7 onwards for *E. coli* and *S. enterica*.

6.2.1 Response surface methodology study for statistical optimization of protective agents on *E. coli* as target analyte

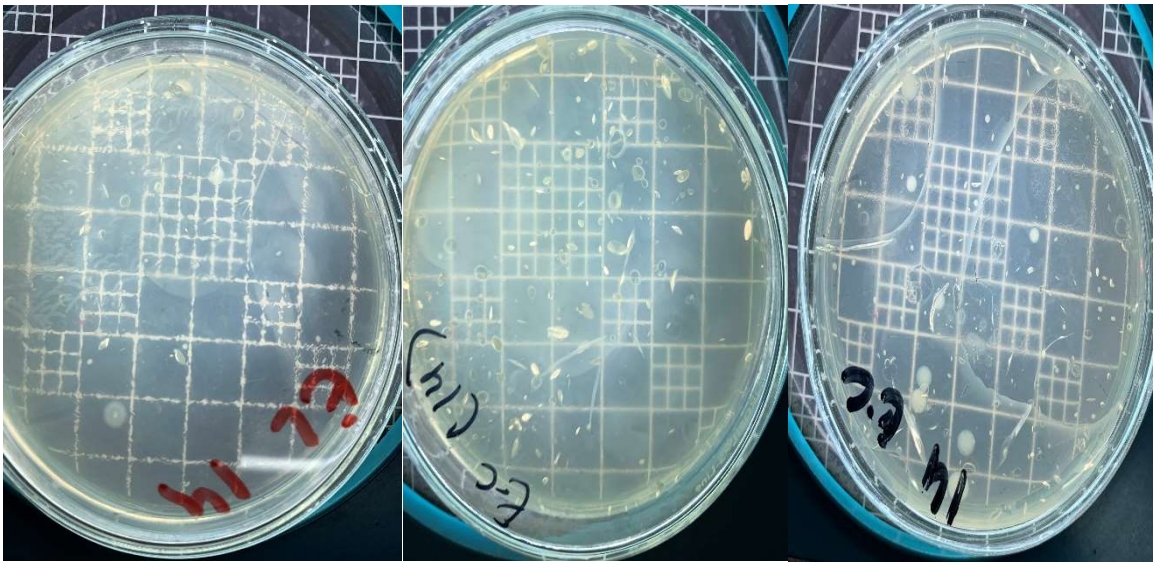
E. coli MTCC 1610^T was propagated overnight on agar (TSA) and was used at a titre of 2.49 Log_{10} culture as initial inoculum for the RSM study and its survival was monitored for 28 days with 5%, 10% and 15% concentration of glucose, glycerol, trehalose and 0, 2.5% and 5% of BSA. Details of the BBD model with actual recovery and predictive values of *E. coli* to Log_{10} value for storage temperature at 5±2°C are provided in Table 11. The statistical analysis (ANOVA) of the proposed quadratic model for the survival of *E. coli* at 5±2°C is summarized in Table 12. The model was found to be satisfactory and statistically significant by the p-value (<0.0001), non-significant lack of fit (F value 1.89), and fit statistics (adequate precision of 20.74 > 4) for *E. coli* at 28 days. The RSM study for optimization of protectants was found to be significant as the observed values of R-squared (0.98, 0.95, 0.91, 0.88 and 0.83 at 0, 7, 14, 21 and 28 days, respectively) were higher, demonstrating a strong correlation between the experimental and predicted values. Through the study, VIFs were observed to be 1.00, indicating the orthogonal nature of factors. The signal to noise ratio observed for the protectant study involving *E. coli* at Day 0 (73.162), Day 7 (39.440), Day 14 (30.044), Day 21 (24.921), and Day 28 (20.738) was greater than the desired ratio (4.0) further indicating the accuracy of the proposed model. The calculated values of deviation (actual and predicted values of activities) for Day 0 (1.53%), Day 7 (2.89%), Day 14 (4.03%), and Day 21 (6.20%) were observed within the permitted limits (± 10%), therefore, showing the model to be satisfactory and statistically significant. The calculated values of deviation (actual and predicted values of activities) for Day 28 (11.17%) were observed outside the permitted limits (± 10%). The linear model was found to be satisfactory and statistically significant by the p-value (<0.0500), non-significant lack of fit (F value 1.89), and fit statistics (adequate precision of 20.74 >4). A photographic representation of the viability study results for one of the RSM runs is shown in figure 35. The effect of glucose, trehalose, glycerol & BSA on the survival of *E. coli* is further elaborated by the 3-D surface contour graph (Figure 36), where the maximum response is achieved with a higher percentage of glycerol and decreasing levels of glucose with the fixed variable of 10% trehalose & 2.5% BSA. Glucose and glycerol have been used widely for cryopreservation (224). Glucose is required by microbial cells as a part of

their active growth cycle, whereas glycerol has long proven to be an effective cryoprotectant by penetrating the microbial cell and protecting the cell membrane and cell wall (33). In various studies involving glucose and glycerol as cryoprotectant additives, glycerol is found to provide better survival of microorganisms than glucose at lower temperatures (210,224,225). Glycerol, combined with nutritive supplements like peptone and yeast extract at -20°C, has resulted in 88.87% and 84.35% survival rates of 15 strains of *Enterobacterales,* including a strain of *Escherichia marmotae* (226).

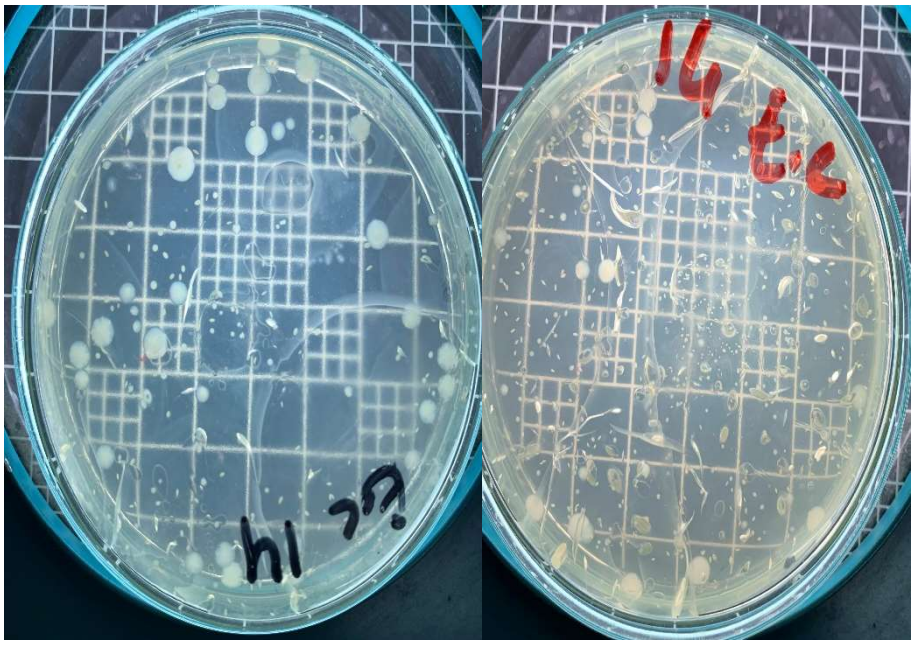
The linear model was found to be satisfactory and statistically significant for *E. coli*. The relationship between the survival rate of *E. coli* MTCC 1610^{T} and the four protectants observed on Day 28 is shown in the equation below (9).

$$Y = 1.56 - 0.46(A) + 0.08(B) + 0.04(C) - 0.29(D) \quad (9)$$

were Y = Response Value (Survival Rate); A = Glucose; B = Glycerol; C = Trehalose; D = BSA with their respective coefficient estimate factors



Day 0 Day 7 Day 14



Day 21 Day 28

Figure 35. Photograph of viability results observed during response surface methodology study for optimization of protective agents on *E. coli* MTCC 1610^{T} at 5±2°C.

Table 11: Box Behnken design for optimization of protectants along with actual and predicted values of *E. coli* MTCC 1610^{T} recovery value converted to Log_{10} value

					Independent variables				Response 1		Response 2		Response 3		Response 4		Response 5	
	A	B	C	D	A:	B:	C:	D:	Day 0		Day 7		Day 14		Day 21		Day 28	
					Glucose	Glycerol	Trehalose	BSA	Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10}(CFU/mL)		Log_{10} (CFU/mL)	
Run					(%)	(%)	(%)	(%)										
	Coded levels				Actual levels				AV	PV	AV	PV	AV	PV	AV	PV	AV	PV
1	-1	0	0	-1	5	10	10	0	2.36	2.35	2.38	2.36	2.34	2.36	2.40	2.36	2.33	2.31
2	+1	0	+1	0	15	10	15	2.5	1.60	1.64	1.62	1.66	1.56	1.59	1.43	1.44	1.26	1.14
3	0	0	0	0	10	10	10	2.5	1.98	1.91	2.02	1.91	1.99	1.88	1.89	1.78	1.66	1.56
4	+1	0	0	-1	15	10	10	0	1.81	1.77	1.83	1.78	1.80	1.75	1.77	1.65	1.53	1.40
5	0	0	-1	-1	10	10	5	0	2.02	2.04	1.99	2.03	2.01	2.04	1.87	2.00	1.74	1.82
6	-1	0	-1	0	5	10	5	2.5	2.14	2.18	2.15	2.16	2.13	2.17	2.06	2.12	1.94	1.98
7	0	+1	+1	0	10	15	15	2.5	1.96	1.93	1.93	1.95	1.88	1.94	1.83	1.84	1.63	1.68
8	0	0	0	0	10	10	10	2.5	1.89	1.91	1.83	1.91	1.81	1.88	1.67	1.78	1.41	1.56
9	0	+1	0	-1	10	15	10	0	2.04	2.06	2.01	2.07	2.03	2.09	1.99	2.05	1.79	1.94
10	0	0	-1	+1	10	10	5	5	1.75	1.74	1.69	1.71	1.67	1.70	1.56	1.55	1.20	1.23
11	+1	0	-1	0	15	10	5	2.5	1.57	1.59	1.54	1.58	1.48	1.57	1.41	1.42	0.85	1.07
12	0	0	0	0	10	10	10	2.5	1.91	1.91	1.92	1.91	1.86	1.88	1.81	1.78	1.69	1.56
13	0	-1	+1	0	10	5	15	2.5	1.94	1.94	1.96	1.94	1.89	1.86	1.78	1.74	1.57	1.52
14	-1	-1	0	0	5	5	10	2.5	2.24	2.21	2.20	2.19	2.14	2.15	2.03	2.09	1.92	1.94
15	-1	+1	0	0	5	15	10	2.5	2.23	2.20	2.25	2.20	2.26	2.23	2.16	2.18	2.08	2.10
16	0	-1	0	-1	10	5	10	0	2.02	2.06	2.01	2.06	2.01	2.02	1.95	1.96	1.75	1.77
17	0	+1	0	+1	10	15	10	5	1.72	1.76	1.68	1.75	1.78	1.75	1.69	1.61	1.36	1.35
18	0	-1	0	+1	10	5	10	5	1.74	1.76	1.69	1.74	1.72	1.68	1.64	1.51	1.28	1.18
19	-1	0	+1	0	5	10	15	2.5	2.22	2.23	2.19	2.23	2.12	2.20	2.07	2.14	1.97	2.06

20	0	0	+1	-1	10	10	15	0	2.08	2.08	2.13	2.11	2.10	2.07	2.01	2.02	1.93	1.89
21	-1	0	0	+1	5	10	10	5	2.06	2.06	2.09	2.03	2.06	2.02	1.98	1.91	1.85	1.72
22	0	0	+1	+1	10	10	15	5	1.79	1.79	1.81	1.78	1.76	1.73	1.51	1.57	1.00	1.31
23	0	-1	-1	0	10	5	5	2.5	1.92	1.89	1.93	1.86	1.96	1.83	1.88	1.72	1.72	1.44
24	+1	0	0	+1	15	10	10	5	1.48	1.47	1.45	1.46	1.26	1.41	0.95	1.21	0.85	0.81
25	+1	-1	0	0	15	5	10	2.5	1.64	1.62	1.62	1.61	1.49	1.54	1.23	1.38	0.60	1.02
26	0	+1	-1	0	10	15	5	2.5	1.90	1.88	1.87	1.87	1.90	1.91	1.73	1.82	1.45	1.61
27	0	0	0	0	10	10	10	2.5	1.90	1.91	1.83	1.91	1.91	1.88	1.89	1.78	1.64	1.56
28	+1	+1	0	0	15	15	10	2.5	1.62	1.61	1.73	1.62	1.82	1.62	1.70	1.48	1.52	1.19
29	0	0	0	0	10	10	10	2.5	1.90	1.91	1.92	1.91	1.90	1.88	1.85	1.78	1.76	1.56

Actual values (AV); predicted values (PV)

Table 12*:* Statistical analysis (ANOVA) by RSM of the proposed model for survival of *E. coli* MTCC 1610^T leading to optimization of protectants when stored at 5±2°C for 28 days

	Day 0	Porb>F	Day 7	Porb>F	Day 14	Porb>F	Day 21	Porb>F	Day 28	Porb>F
Model	**1.31 (Significant)**	**<0.0001**	**1.34 (Significant)**	**< 0.0001**	**1.47 (Significant)**	**< 0.0001**	**2.10 (Significant)**	**< 0.0001**	**3.64 (Significant)**	**< 0.0001**
A	1.04	< 0.0001	1.00	< 0.0001	1.10	< 0.0001	1.48	< 0.0001	2.50	< 0.0001
B	0.0001	0.7692	0.0003	0.7558	0.0176	0.0929	0.0290	0.1368	0.0817	0.1143
C	0.0070	0.0085	0.0184	0.8046	0.0021	0.5485	0.0012	0.7569	0.0176	0.4538
D	0.2670	< 0.0001	0.3136	< 0.0001	0.3468	< 0.0001	0.5896	< 0.0001	1.04	< 0.0001
R^2	0.9847		0.9483		0.9141		0.8771		0.8330	
Adj. R^2	0.9821		0.9397		0.8998		0.8566		0.8051	
Lack of fit	Not Significant		Not Significant		Not Significant		Not Significant		Not Significant	

Probability value of Fisher's variance ratio (Prob>F), coefficient of determination (R^2)

The concentration of CH, SPH and FSH is represented by independent variables A, B and C.

The effect of variables in squared terms (A^2, B^2, C^2)

Interactive effect of the variables (AB, AC, BC)

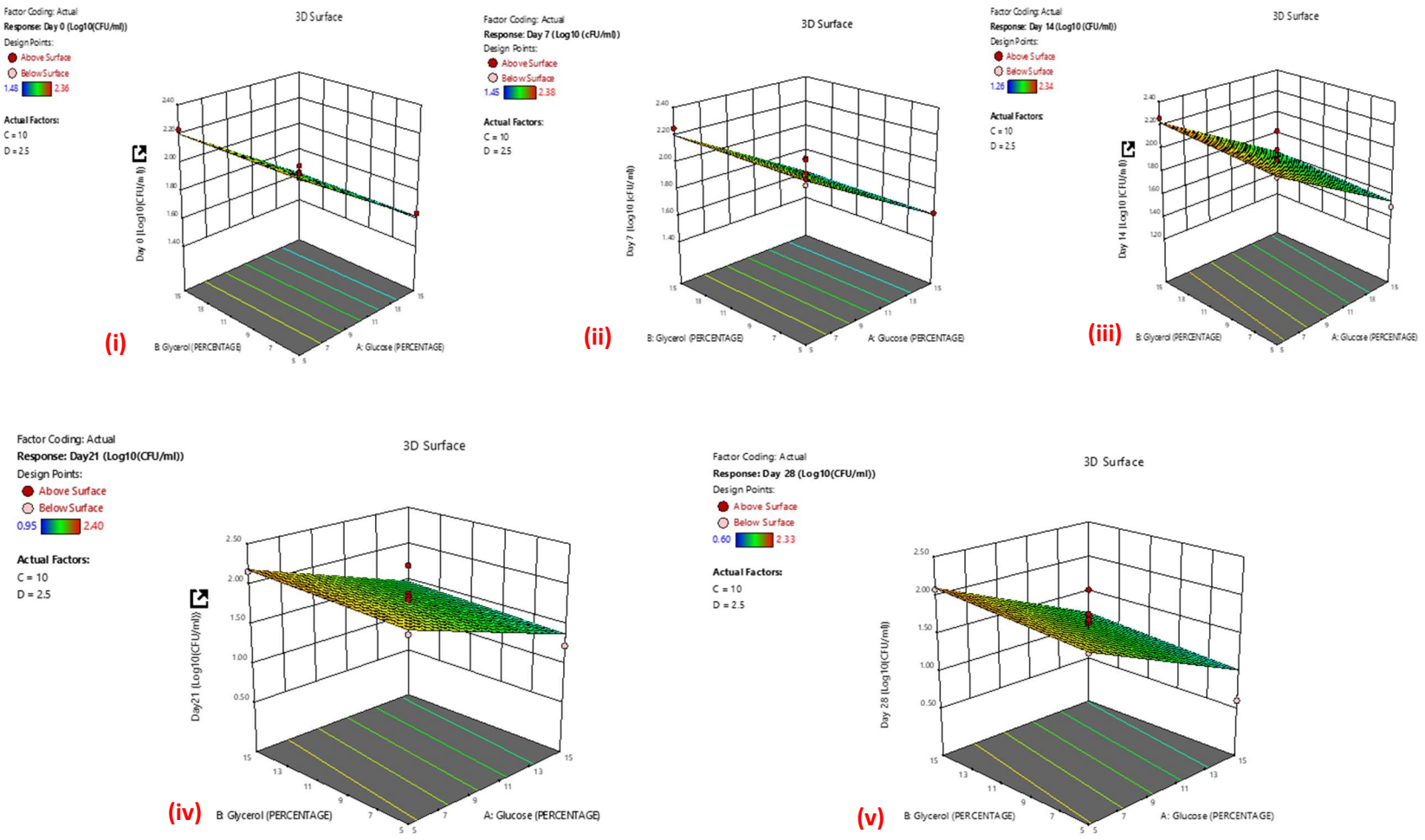


Figure 36. 3-D Contours showing the effect of glucose (A), glycerol (B), trehalose (C), and bovine serum albumin (D) on the survival of *E. coli* MTCC 1610^{T} at 5±2°C on (i) Day 0, (ii) Day 7, (iii) Day 14, (iv) Day 21 and (v) Day 28

6.4.2 Response surface methodology study for statistical optimization of protective agents on *S. enterica* as target analyte

Concentration selected for glucose, trehalose, and glycerol were 5%, 10% & 15% whereas 0, 2.5% & 5% concentration level was selected for BSA in the RSM study of protectants. *S. enterica* MTCC 660^T was propagated overnight on agar (TSA) and was used at a titre of 2.51 Log_{10} culture as initial inoculum for the RSM study, and its survival was monitored for a period of 28 days. Details of the BBD model with actual recovery and predictive values of *S. enterica* to Log_{10} value for storage temperature at 5±2°C are provided in Table 13. The statistical analysis using ANOVA of the proposed model for survival of *S. enterica* is elaborated in Table 14. The RSM study for optimization of protectants was found to be significant as the observed values of R-squared were >0.92 at Day 0, Day 7, Day 14 & Day 28, and > 0.86 at Day 21, demonstrating a strong correlation among the experimental and predicted values. The model was deemed satisfactory and statistically significant, as indicated by the p-value, the non-significant lack of fit, and the fit statistics (Table 14). A p-value of less than 0.0001, which is less than 0.05, implies that the model terms are significant. Additionally, a lack of fit value of 2.12, observed in the Day 28 response, suggests that the lack of fit is not significant. Throughout the study, Variance Inflation Factors (VIFs) were observed to be 1.00, indicating the orthogonal nature of the factors.

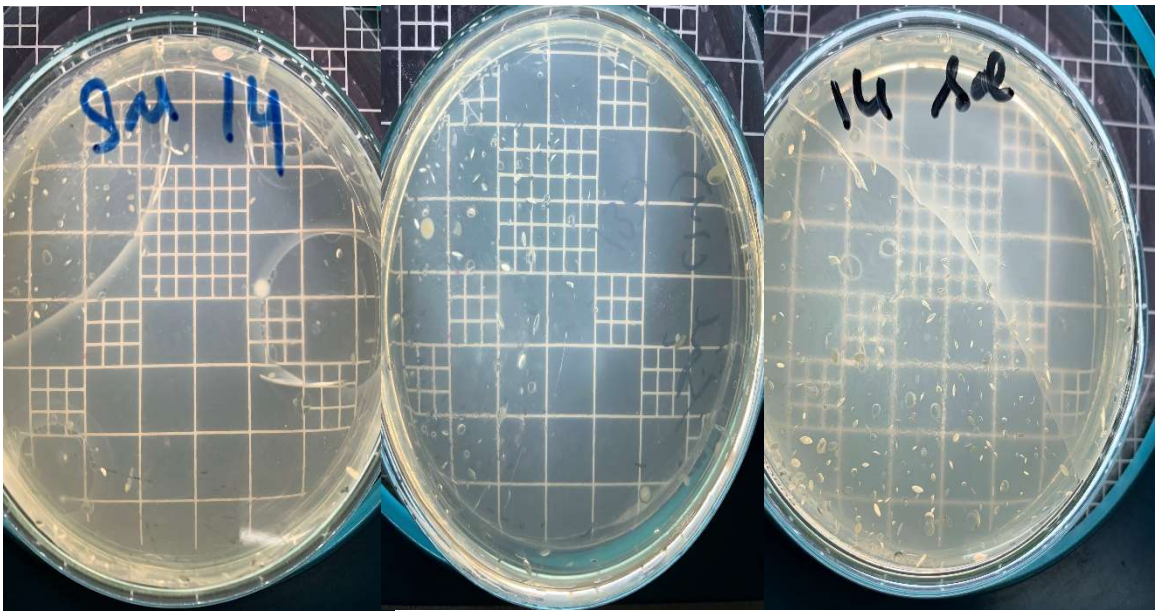
The signal to noise ratio for the RSM protectant study at Day 0 (30.370), Day 7 (27.358), Day 14 (18.011), Day 21 (20.362), and Day 28 (16.473) was greater than the desired ratio (4.0), further indicating the accuracy of the proposed model. The calculated values of deviation (actual and predicted values of activities) for Day 0 (6.06%) and Day 7 (9.33%), were observed within the permitted limits (± 10%), therefore, showing the model to be satisfactory and statistically significant. The calculated values of deviation (actual and predicted values of activities) for Day 14 (12.42%), Day 21 (21.01%), and Day 28 (17.73%) were observed outside the permitted limits (± 10%). RSM study is applied in the optimization of metabolites produced by microbial cells. In the microbiology optimization studies utilizing RSM which are available in scientific literature, have chemical analysis of

microbial metabolites as a response rather than viability count of the microbial cells (191). Counting of colonies involves a higher standard deviation due to the non-homogeneous presence of micro-organisms, even in a simple matrix like water (191,227,228). The measurement uncertainty of microbiological testing is generally higher than that of chemical testing. Both methods differ in terms of precision. Therefore, the ±10% standard deviation (SD) limit applies to chemical analysis, rather than viability count (229).

A photographic representation of the viability study results for one of the RSM runs is represented in figure 37. The impact of glucose, trehalose, glycerol, and Bovine Serum Albumin (BSA) on the survival of *S. enterica* is further illustrated by the 3-D surface contour graphs (Figure 38). The graph shows that the maximum response is achieved with a higher percentage of glycerol and decreasing levels of glucose while keeping the variables of trehalose and BSA fixed at 10 and 2.5%, respectively. The optimal conditions for the survival of *S. enterica* are achieved with a higher concentration of glycerol and a lower concentration of glucose in this study. This can be attributed to the role of glucose as an energy provider for cells in the active phase, rather than the dormant phase (230). In *Salmonella* survival studies involving glycerol and glucose as humectant or polyol and sugar, respectively, gene expression involving features of heat and acid resistance, virulence, filamentation, biofilm formation, and survival in low water activity increases in the presence of glycerol as compared to glucose at increasing concentration (119,231–234). In the dormant phase, microbial cells need to minimize their metabolic activity for survival, and this is facilitated by lower temperatures. Therefore, under such conditions, glycerol serves as a superior cryoprotective agent compared to glucose (235). Glycerol protects cell components at lower temperatures and aids in microbial survival (33,224).

The linear model was found to be satisfactory and statistically significant for *S. enterica* at Day 0, Day 7, and Day 21. However, for Day 14 and Day 28, *S. enterica* demonstrated quadratic relationship and the quadratic model was proved to be both satisfactory and statistically significant on these days. The relationship between the survival rate of S. enterica and the four protectants, as observed on Day 28, is illustrated in Equation (11).

$$Y = 1.23 - 0.70(A) - 0.30(B) - 0.26(C) - 0.15(D) + 0.04(AB) - 0.07(AC) + 0.09(AD) + 0.01(BC) - 0.05(BD) - 0.08\,(CD) - 0.04(A)^2 - 0.16\,(B)^2 - 0.21(C)^2 - 0.14(D)^2 \quad \ldots\ldots\ldots\ldots (11)$$



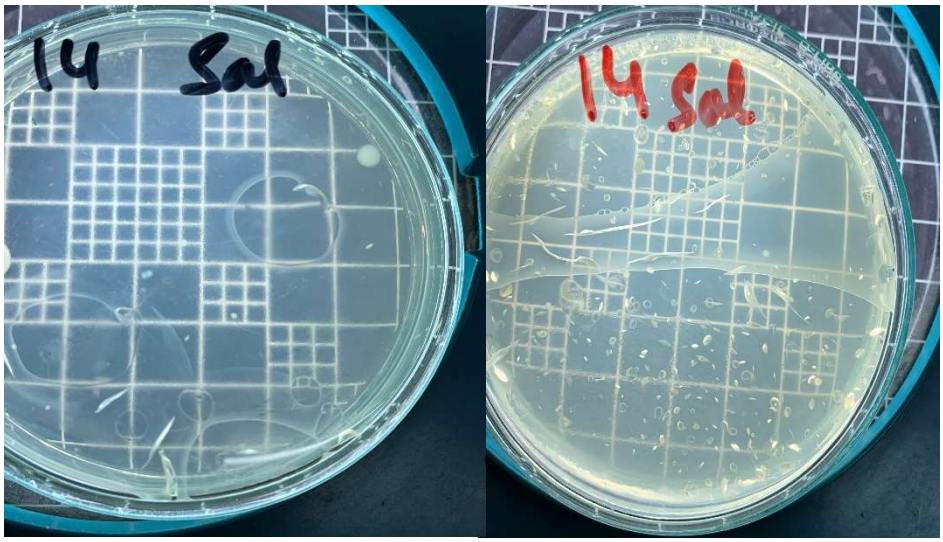


Figure 37. Photograph of viability results observed during response surface methodology study optimization of protective agents on *S. enterica* subspp. Arizonae MTCC 660^T at 5±2°C.

Table 13: Box Behnken design for optimization of protectants along with actual and predicted values of *S. enterica* subspp. Arizonae MTCC 660^{T} recovery value converted to Log$_{10}$ value.

	Independent variables								Response 1		Response 2		Response 3		Response 4		Response 5	
	A	**B**	**C**	**D**	**A: Glucose (%)**	**B: Glycerol (%)**	**C: Trehalose (%)**	**D: BSA (%)**	**Day 0 Log$_{10}$(CFU/mL)**		**Day 7 Log$_{10}$(CFU/mL)**		**Day 14 Log$_{10}$(CFU/mL)**		**Day 21 Log$_{10}$(CFU/mL)**		**Day 28 Log$_{10}$(CFU/mL)**	
Run	**Coded levels**				**Actual levels**				**AV**	**PV**	**AV**	**PV**	**AV**	**PV**	**AV**	**PV**	**AV**	**PV**
1	-1	0	0	-1	5	10	10	0	2.15	1.96	2.12	1.99	2.19	2.05	2.13	1.93	2.06	1.99
2	+1	0	+1	0	15	10	15	2.5	1.08	1.00	0.78	0.72	0.30	0.27	0.60	0.19	0.00	-0.03
3	0	0	0	0	10	10	10	2.5	1.54	1.44	1.45	1.33	1.23	1.18	1.08	1.03	1.20	1.23
4	+1	0	0	-1	15	10	10	0	1.30	1.20	1.18	1.01	1.00	0.84	0.90	0.71	0.48	0.42
5	0	0	-1	-1	10	10	5	0	1.54	1.64	1.64	1.62	1.45	1.41	1.34	1.55	1.38	1.20
6	-1	0	-1	0	5	10	5	2.5	1.92	1.88	1.99	1.94	1.83	1.94	1.73	1.88	1.69	1.88
7	0	+1	+1	0	10	15	15	2.5	1.20	1.21	1.00	0.99	0.48	0.37	0.48	0.49	0.30	0.32
8	0	0	0	0	10	10	10	2.5	1.41	1.44	1.34	1.33	1.26	1.18	1.30	1.03	1.34	1.23
9	0	+1	0	-1	10	15	10	0	1.34	1.41	1.20	1.29	0.90	1.03	0.90	1.01	0.60	0.82
10	0	0	-1	+1	10	10	5	5	1.36	1.36	1.34	1.28	1.20	1.02	1.15	0.99	1.23	1.08
11	+1	0	-1	0	15	10	5	2.5	1.08	1.12	0.70	0.96	0.78	0.94	0.70	0.66	0.30	0.62
12	0	0	0	0	10	10	10	2.5	1.18	1.44	1.00	1.33	1.00	1.18	0.95	1.03	1.08	1.23
13	0	-1	+1	0	10	5	15	2.5	1.45	1.55	1.34	1.42	1.18	1.15	1.08	1.11	0.90	0.91
14	-1	-1	0	0	5	5	10	2.5	2.02	1.99	2.04	2.03	1.99	2.04	2.01	1.95	1.95	2.09
15	-1	+1	0	0	5	15	10	2.5	1.68	1.65	1.63	1.60	1.57	1.57	1.46	1.34	1.48	1.40
16	0	-1	0	-1	10	5	10	0	1.72	1.75	1.62	1.72	1.51	1.52	1.40	1.62	1.32	1.34
17	0	+1	0	+1	10	15	10	5	1.11	1.13	0.85	0.94	0.30	0.37	0.30	0.45	0.30	0.44
18	0	-1	0	+1	10	5	10	5	1.48	1.47	1.38	1.37	1.15	1.09	1.04	1.06	1.20	1.13
19	-1	0	+1	0	5	10	15	2.5	1.78	1.76	1.75	1.69	1.68	1.60	1.62	1.41	1.66	1.50
20	0	0	+1	-1	10	10	15	0	1.45	1.52	1.28	1.38	0.85	1.05	0.70	1.08	0.78	0.86
21	-1	0	0	+1	5	10	10	5	1.62	1.68	1.58	1.64	1.40	1.46	1.49	1.36	1.53	1.52
22	0	0	+1	+1	10	10	15	5	1.26	1.24	0.95	1.03	0.30	0.37	0.00	0.52	0.30	0.40

23	0	-1	-1	0	10	5	5	2.5	1.70	1.67	1.62	1.67	1.46	1.48	1.40	1.58	1.53	1.44
24	+1	0	0	+1	15	10	10	5	1.00	0.92	0.85	0.67	0.30	0.35	0.00	0.14	0.30	0.30
25	+1	-1	0	0	15	5	10	2.5	1.23	1.23	1.15	1.06	1.00	1.02	0.90	0.73	0.60	0.60
26	0	+1	-1	0	10	15	5	2.5	1.34	1.33	1.28	1.23	1.11	1.05	1.00	0.96	0.90	0.82
27	0	0	0	0	10	10	10	2.5	1.48	1.44	1.38	1.33	1.23	1.18	1.20	1.03	1.32	1.23
28	+1	+1	0	0	15	15	10	2.5	0.90	0.89	0.60	0.62	0.30	0.27	0.00	0.12	0.30	0.08
29	0	0	0	0	10	10	10	2.5	1.49	1.44	1.45	1.33	1.20	1.18	1.15	1.03	1.23	1.23

Actual values (AV); predicted values (PV)

Table 14*:* Statistical analysis (ANOVA) by RSM of the proposed model for survival of *S. enterica* subspp. Arizonae MTCC 660^T leading to optimization of protectants when stored at 5±2°C over a period of 28 days.

	Day 0	Porb>F	Day 7	Porb>F	Day 14	Porb>F	Day 21	Porb>F	Day 28	Porb>F
	2.37	**<0.0001**	**3.95**	**< 0.0001**	**7.22**	**< 0.0001**	**7.25**	**< 0.0001**	**8.54**	**< 0.0001**
Model	**(Significant)**		**(Significant)**		**(Significant)**		**(Significant)**		**(Significant)**	
A	1.75	< 0.0001	2.85	< 0.0001	4.06	< 0.0001	4.49	< 0.0001	5.87	< 0.0001
B	0.3434	< 0.0001	0.5590	< 0.0001	1.10	< 0.0001	1.13	< 0.0001	1.09	< 0.0001
C	0.0432	0.0257	0.1801	0.0022	0.7701	0.0022	0.6721	0.0009	0.7957	0.0002
D	0.2324	< 0.0001	0.3640	< 0.0001	0.8802	< 0.0001	0.9577	0.0001	0.2581	0.0131
A^2	-	-	-	-	0.0751	0.0665	-	-	0.0081	0.6227
B^2	-	-	-	-	0.0284	0.2412	-	-	0.1590	0.0426
C^2	-	-	-	-	0.0748	0.0669	-	-	0.2802	0.0103
D^2					0.0875	0.0497			0.1347	0.0594
AB	-	-	-	-	0.0196	0.3266	-	-	0.0072	0.6419

AC	-	-	-	-	0.0272	0.2507	-	-	0.0182	0.4628
AD					0.0020	0.7487			0.0306	0.3444
BC	-	-	-	-	0.0306	0.2245	-	-	0.0002	0.9343
BD			-	-	0.0144	0.3982			0.0081	0.6226
CD					0.0225	0.2944			0.0272	0.3718
R^2	0.9282		0.9149		0.9645		0.8647		0.9502	
Adj. R^2	0.9162		0.9007		0.9291		0.8422		0.9003	
Lack of fit	Not Significant		Not Significant		Not Significant		Not Significant		Not Significant	

Probability value of Fisher's variance ratio (Prob>F), coefficient of determination (R^2)
The concentration of glucose, glycerol, trehalose and BSA is represented by independent variables A, B, C and D.
The effect of variables in squared terms (A^2, B^2, C^2, D^2)
Interactive effect of the variables (AB, AC, AD, BC, BD, CD)

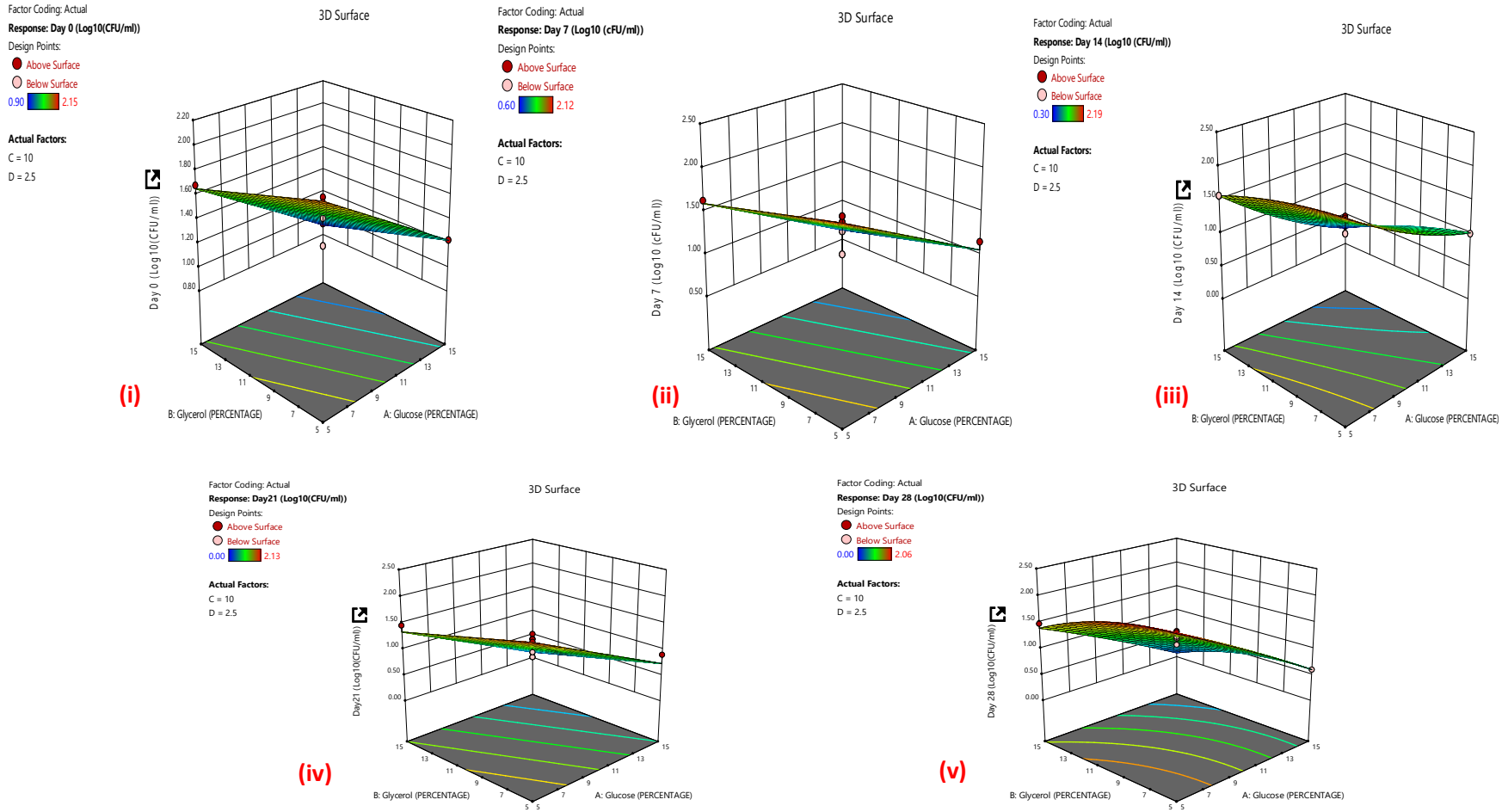


Figure 38. 3-D Contours showing the effect of glucose(A), glycerol (B), trehalose (C), and bovine serum albumin (D) on the survival of *S. enterica* MTCC 660^{T} at 5±2°C on (i) Day 0, (ii) Day 7, (iii) Day 14, (iv) Day 21 and (v) Day 28

6.5 Study of encapsulation agents

Preservation of probiotics has been widely studied for target delivery and avoiding gastric conditions by employing encapsulation techniques (60). Sodium-CMC and gelatin have been studied for microencapsulation of probiotics or bioactives by creating water in water emulsion, which is soluble in water (236,237). Gelatin has also been used to preserve microorganisms during transportation (34,238). Guar gum has been used in the formation of hydrogels and films exhibiting properties like thickening, water solubility, pH stability, gelling, and binding properties (239). For the encapsulation step, CMC, gelatin & guar gum were evaluated for consistency when kept at 45°C for 30 mins (Figure 39), followed by disc or pellet formation by drop method (Figure 40) and drying in a laminar for 3 h. The formed pellet was added to distilled water, and the solubility of the pellet was assessed. Based on the results shown in Table 15, 3% gelatin and 2% CMC were chosen for the encapsulation step, along with the elimination of guar gum as it was leaving residues after dissolution in water (Figure 41).

Table 15: Assessment of encapsulating agents based on physical observations

CMC (%)	Guar Gum (%)	Gelatin (%)	Observations		
			Consistency (45°C @ 30 mins)	**State** (Drying in Laminar for 3 h)	**Solubility in water**
1	1	1	Runny	Liquid	Dissolves completely
1	1	2	Runny	Liquid	Dissolves completely
1	1	3	Runny	Liquid	Dissolves completely
1	2	3	Thick	Semi-Solid	Longer time to dissolves in water
0	2	3	Thick	Semi-Solid	Leaves residue post dissolution in water
0	3	3	Thick	Semi-Solid	Insoluble in water
0	4	4	Thick	Solid	Insoluble in water
2	1	3	Thick	Semi-Solid	Leaves residue post dissolution in water
2	0	3	Thick	Solid	Dissolves completely

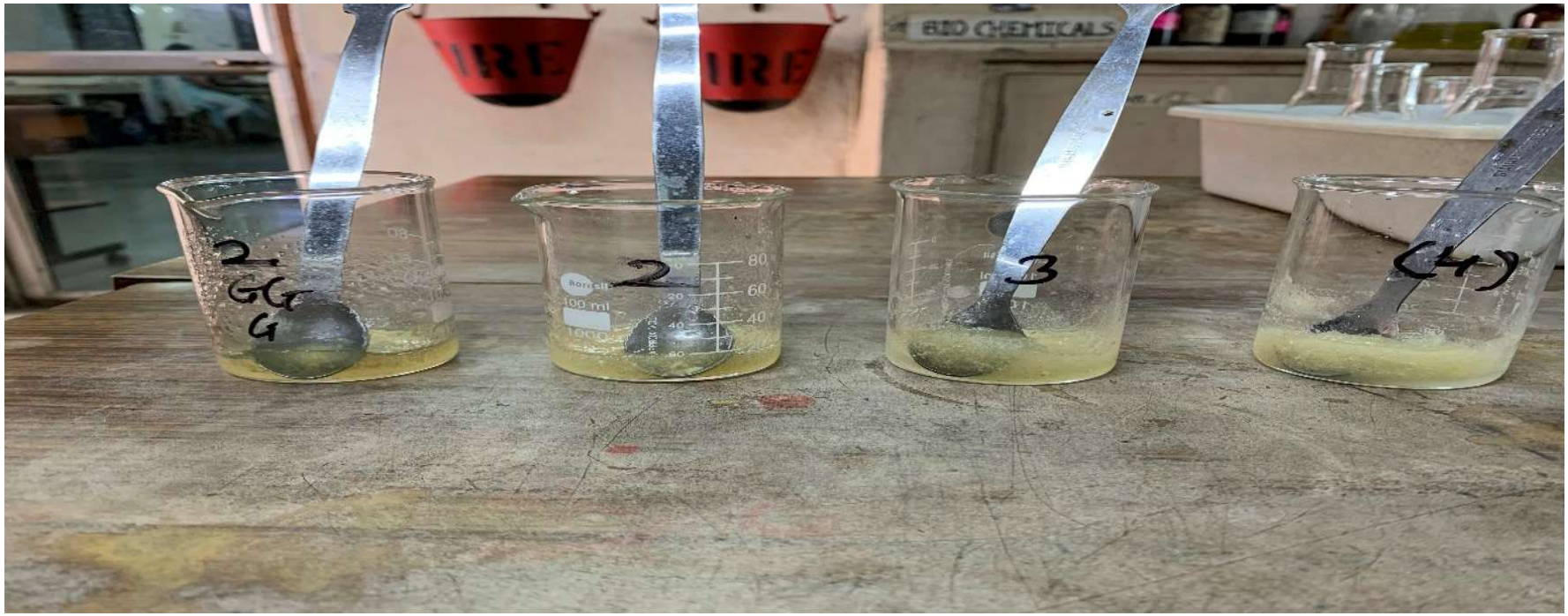


Figure 39. Different concentrations of CMC, guar gum and gelatin for physical assessment of consistency, drying time and solubility in water.

Figure 40. Pellet or disc formation using the drop method to assess different concentrations of CMC, guar gum and gelatin

Figure 41. Dissolution of pellets formed using different concentrations of CMC, guar gum and gelatin in distilled water

6.6 Formulation steps to create quantitative reference material of *E. coli* and *S. enterica*

The formulation and process for preparing quantitative RM are depicted in Figure 42, while the final product is illustrated in figure 43. The quantitative RM for both microorganisms was prepared in three batches and stored at room temperature (31±5°C), refrigeration, and freezing conditions for homogeneity and stability studies.

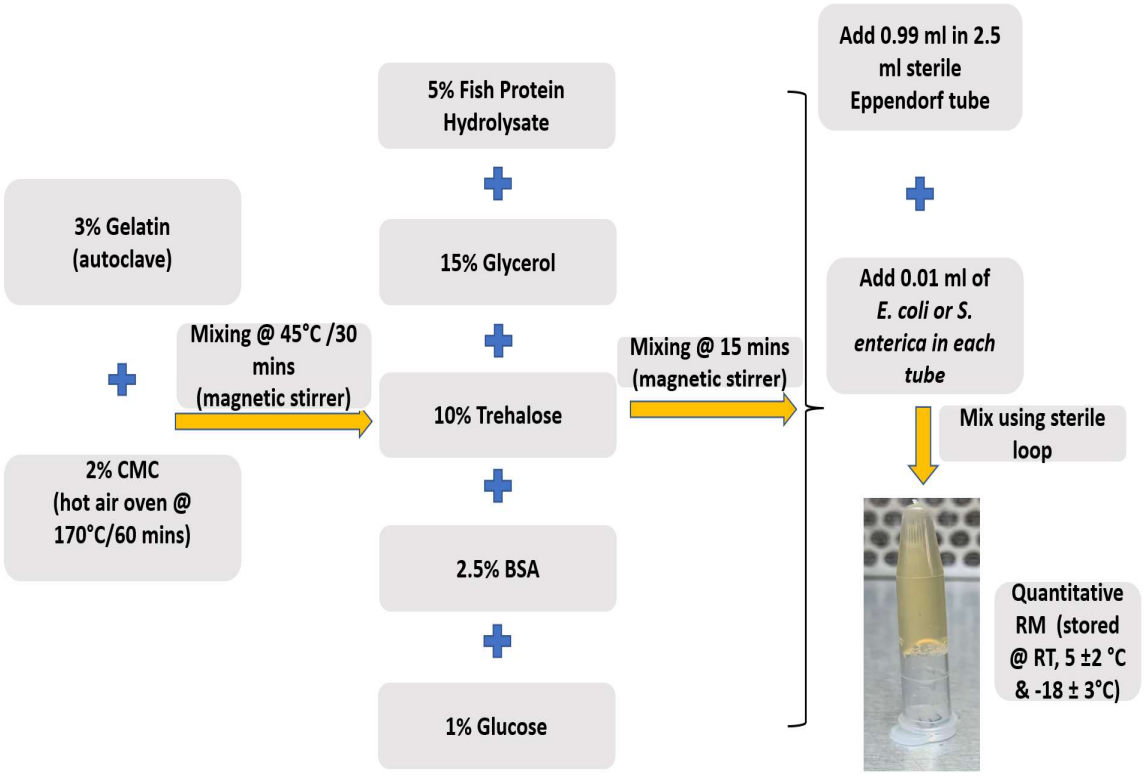


Figure 42. Encapsulation procedure used in the development of quantitative reference material formulation of *E. coli* MTCC 1610^T *and S. enterica* subspp. Arizonae MTCC 660^T

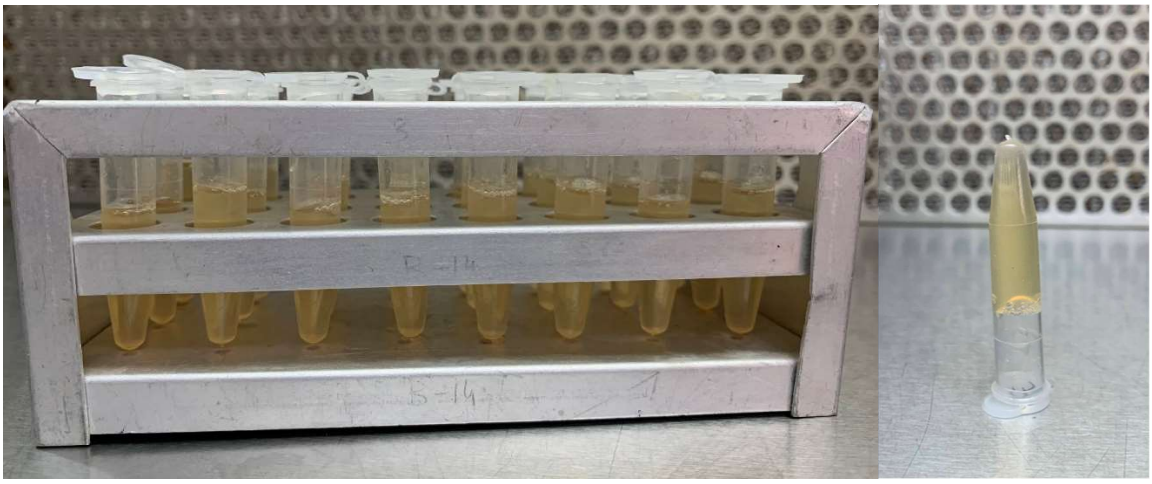


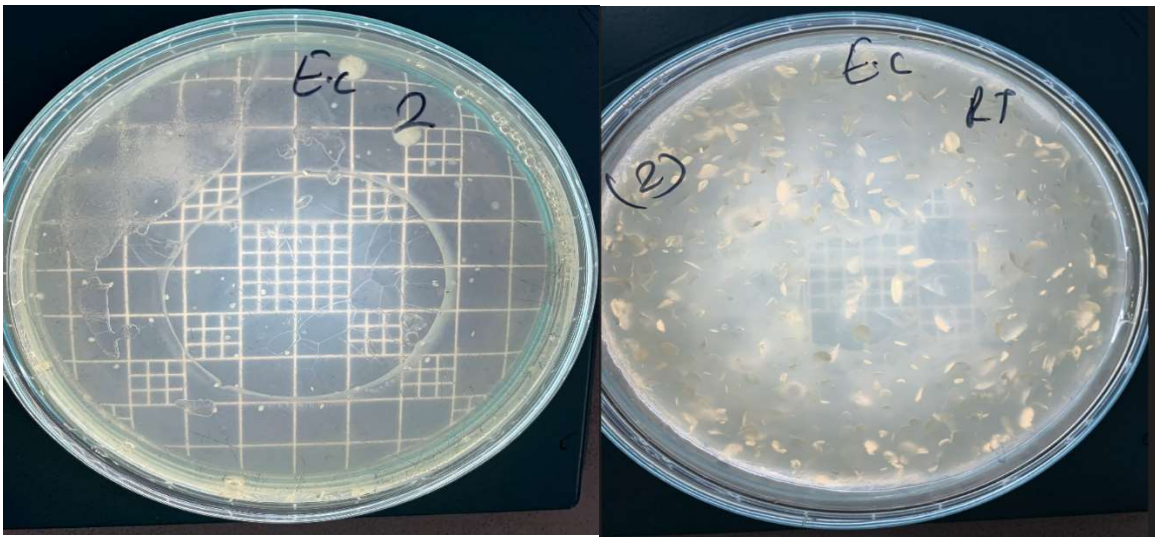
Figure 43. Photograph of the final prepared quantitative reference materials

6.7 Determination of homogeneity and stability

6.7.1 Homogeneity and stability study of *E. coli* based quantitative reference materials

The produced quantitative RM of *E. coli* was stored at room temperature (31±5°C), refrigeration temperature (5±2°C), and freezing temperature (-18±3°C). Room temperature was selected to assess the performance of the product during transportation. The standard deviation (SD) was observed to be 0.19 after 7 days, whereas after Day 14 of storage at room temperature, due to overgrowth of *E. coli*, SD was outside the acceptable limit. Hence, the transportation of the product should not take more than 7 days (Figures 44 and 45). Under refrigeration conditions, the produced quantitative RM showed 0.07 SD for 30 days and lost viability on day 60 (Figures 46 and 47). At freezing conditions, the maximum viability achieved for the produced quantitative RM was 5 months or 150 days (Figure 48). SD of 0.18 was observed up to 4 months, and 0.33 SD at 5 months storage period. There was no viability observed for *E. coli* after 6 months of storage. One way ANOVA was performed on the viability results (of 5 months) to assess the homogeneity where the F_{stat} (0.08) < $F_{critical}$ (3.68) and p-value (0.92) > 0.05 indicated weak evidence against the null hypothesis and failure in rejection of the null hypothesis due to no statical difference and

reaffirms that the samples are homogeneous in nature (Table 16). The assigned or expected value of the prepared quantitative RM for *E. coli*, based on readings observed from day 0 to 120, was calculated to be 2.41 (median value). This calculation includes a maximum acceptable standard deviation (SD) of 0.35 Log_{10} (according to ISO 33405: 2024). The expected range is 2.41 ± 0.35 Log10 (2.06 – 2.76). For the assigned value calculation, readings up to 120 days were selected instead of 150 days, as the SD at 150 days was 0.33, very close to the threshold limit of 0.35 Log_{10}.



Day 0 **Day 7**

Figure 44. Photograph of viability study conducted for produced quantitative RM of *E. coli* MTCC 1610^T stored at room temperature of 31±5°C.

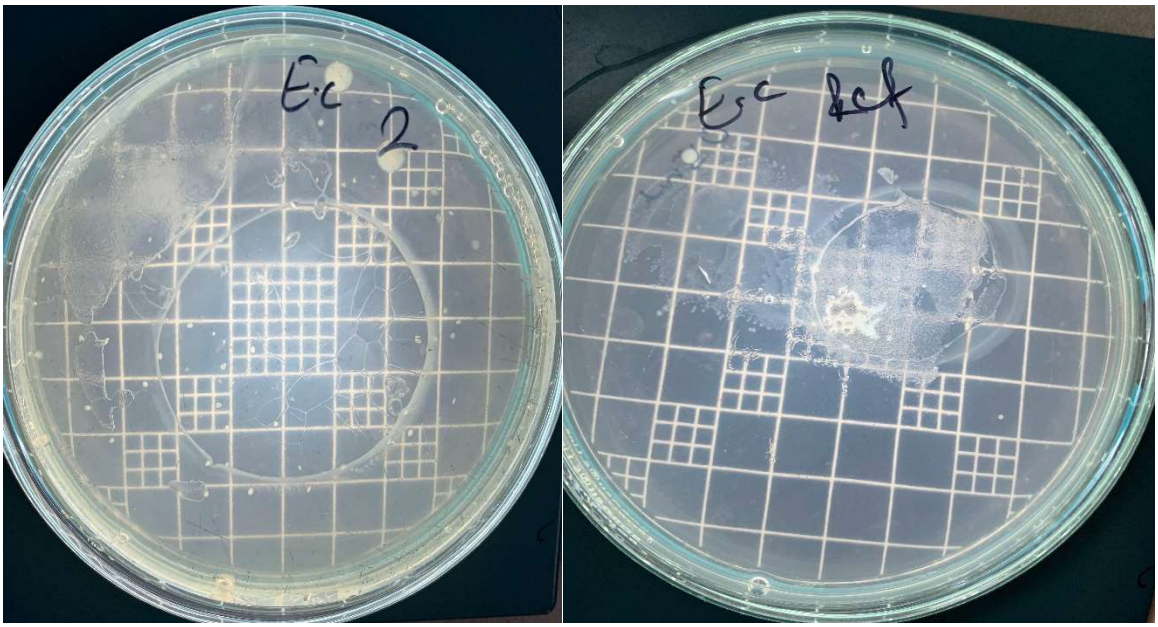


Figure 45. Photograph of viability study conducted for produced quantitative RM of *E. coli* MTCC 1610^{T} stored at 5±3°C.

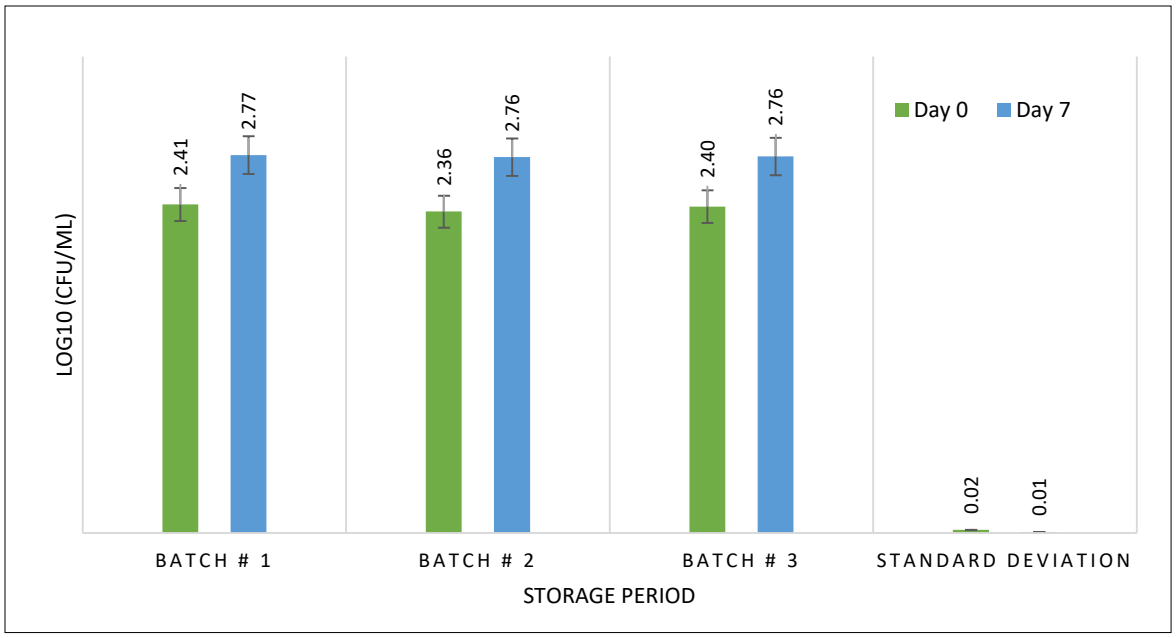


Figure 46. Viability study data of *E. coli* MTCC 1610^{T} quantitative reference material stored at room temperature (31±5°C).

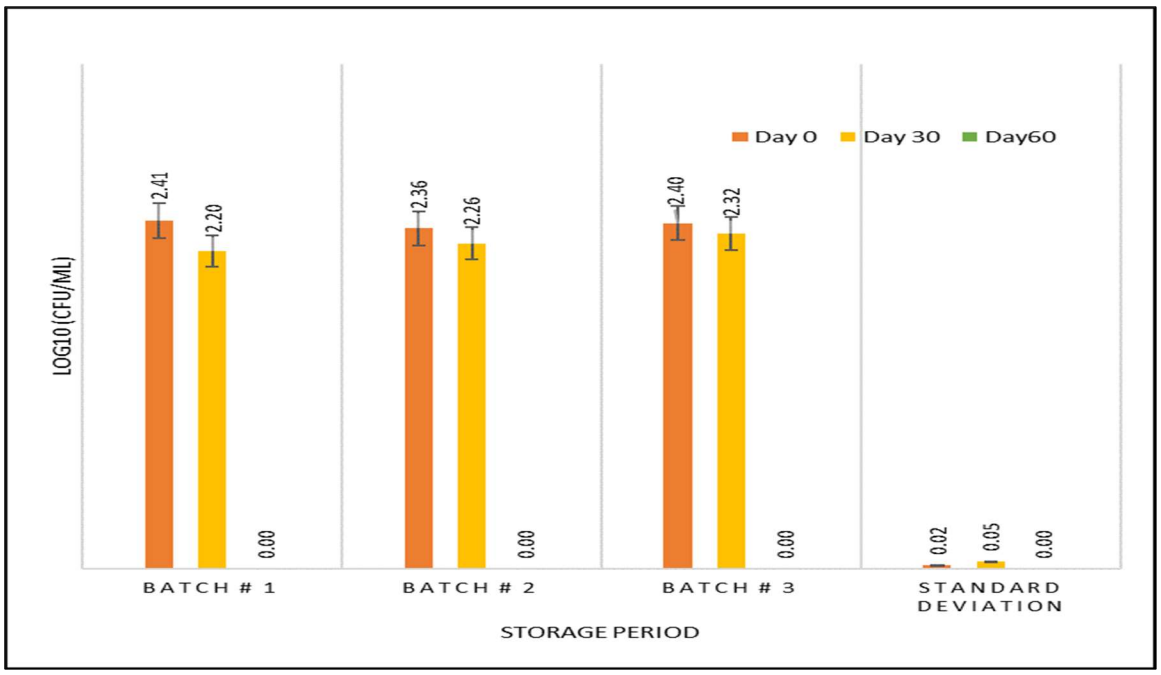


Figure 47. Viability study data of *E. coli* MTCC 1610^{T} quantitative reference material stored at refrigeration conditions (5±3°C).

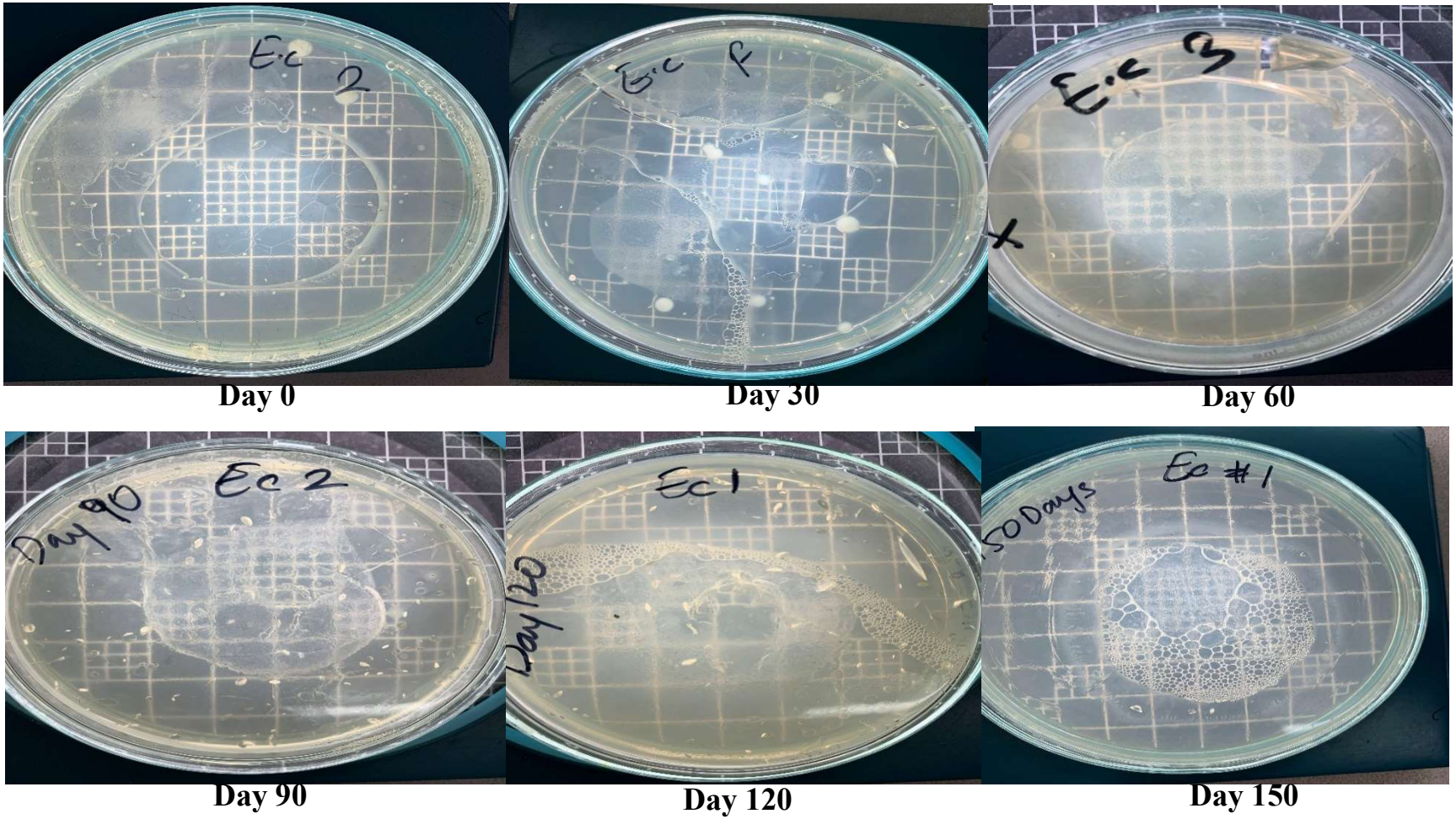


Figure 48. Photograph of viability study conducted for produced quantitative RM of *E. coli* MTCC 1610^{T} stored at -18±3°C.

Table 16: Viability study data converted to logarithmic value for 180 Days and one-way ANOVA analysis result for quantitative reference material of *E. coli* MTCC 1610^{T} at -18±3°C till 150 Days

	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180
Batch # 1	2.41	2.68	2.23	2.26	2.47	1.75	0.00
Batch # 2	2.36	2.70	2.11	2.41	2.41	1.77	0.00
Batch # 3	2.40	2.63	2.18	2.22	2.50	1.43	0.00
Average Count	2.39	2.67	2.17	2.26	2.47	1.75	0.00
Standard Deviation (SD)	0.02	0.03	0.05	0.08	0.04	0.16	0.00
Overall SD			0.33*				- -

Groups	*Count*	*Sum*	*Average*	*Variance*
Batch # 1	6	13.80546	2.30091	0.097952
Batch # 2	6	13.76184	2.293639	0.101007
Batch # 3	6	13.36014	2.22669	0.180693

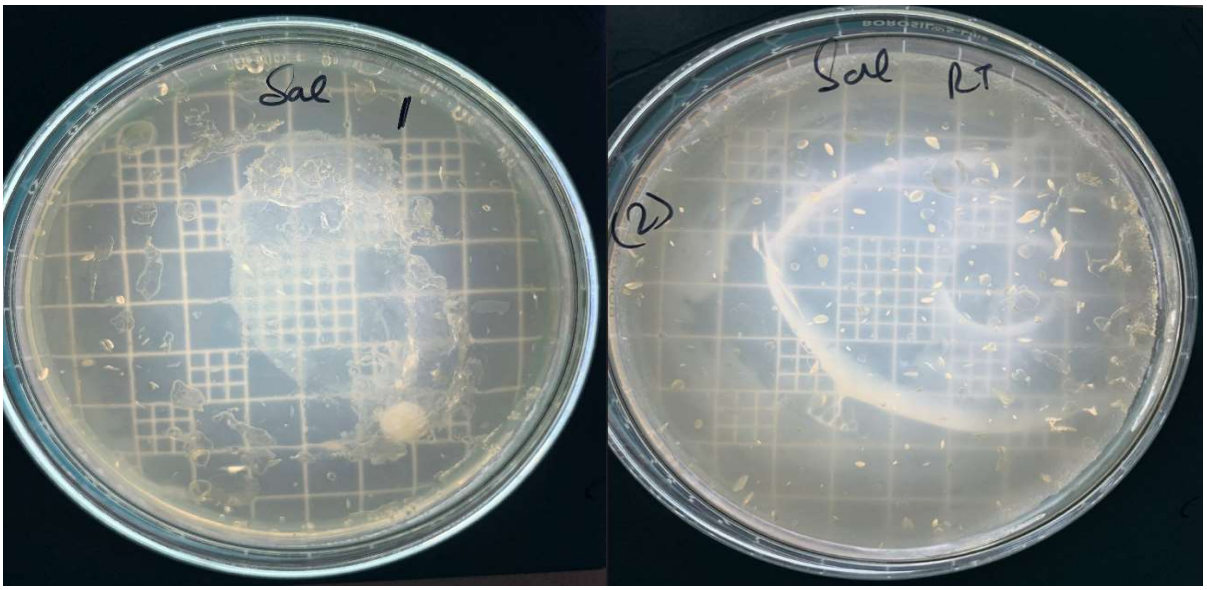
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.020088	2	0.010044	0.079366	0.924087	3.68232
Within Groups	1.898263	15	0.126551			
Total	1.918351	17				

* SD calculated till 150 days as it was >0.35 Log_{10} at 180 days

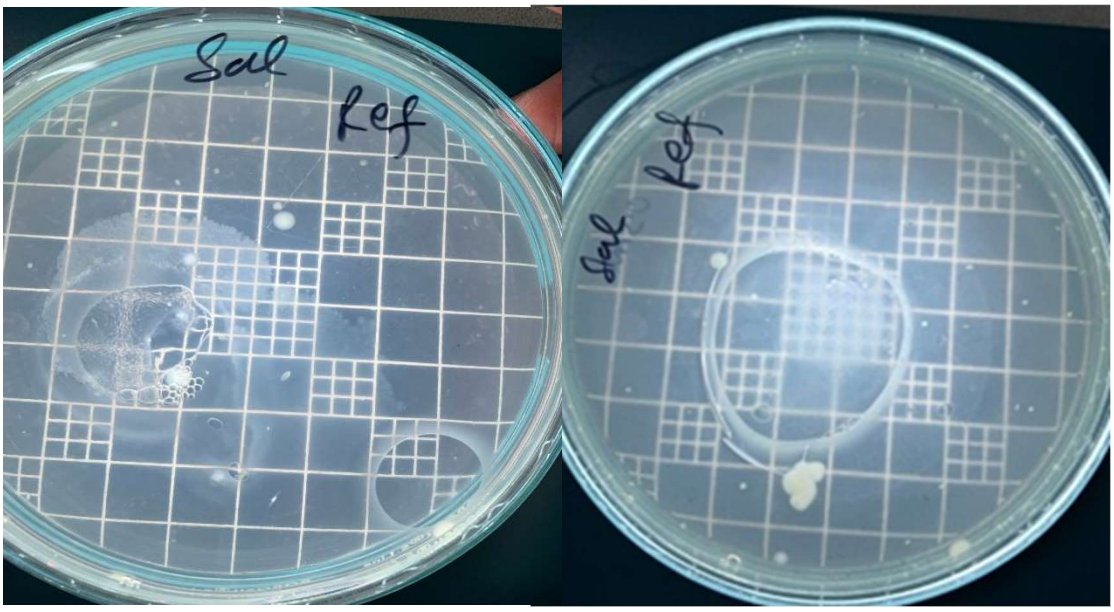
6.7.2 Homogeneity and stability study of *S. enterica* based quantitative reference materials

The standard deviation (SD) was monitored for three different batches of the produced quantitative RM (65). As an internal control, the SD of repeatability and reproducibility of the produced RM was set to be less than or equal to 0.35 Log_{10}. According to the binomial distribution, to achieve a zero probability of defective units at 10% of defective units per lot or 10 sample units tested, the value is 0.35 (240,241). The SD was observed to be 0.04 after 7 days of storage at room temperature. However, after Day 14, the SD exceeded the acceptable limit due to overgrowth (Figures 49 and 50). Therefore, the transportation of the product should not exceed 7 days. Under refrigeration conditions, the produced quantitative RM showed an SD of 0.18 for 30 days and lost viability at 60 days (Figures 51 and 52). Under freezing conditions, the maximum viability achieved for the produced quantitative RM was 5 months. The SD of 0.14 was observed up to 4 months, and an SD of 0.42 was observed at the 5-month storage period (Table 17). Hence, the produced quantitative RM of *S. enterica* has an acceptable shelf life of 4 months or 120 days (Figure 53). A one-way ANOVA was performed on the viability results to assess homogeneity. The F-statistic (0.04) was less than the F-critical value (3.89), the p-value (0.96) was greater than 0.05, and the overall mean Log_{10} variation of the counts between the groups (0.001) was less than or equal to 0.1, which indicates no statistical difference. These results provide limited evidence against the null hypothesis. The failure to reject the null hypothesis suggests that the samples remain homogeneous over 4 months (Table 17). The assigned or expected value of the prepared quantitative RM for *Salmonella enterica* based on the readings observed from day 0 to 120 was calculated to be 2.31 (median value). This calculation includes a maximum acceptable standard deviation (SD) of 0.35 Log_{10} (65). Consequently, the expected range is 2.31 ± 0.35 Log_{10} (corresponding to a confidence interval of 1.96 – 2.66).



Day 0 **Day 7**

Figure 49. Photograph of viability study conducted for produced quantitative RM of *S. enterica* subspp. Arizonae MTCC 660^{T} stored at room temperature of 31±5°C.



Day 7 **Day 30**

Figure 50. Photograph of viability study conducted for produced quantitative reference material of *S. enterica* subspp. Arizonae MTCC 660^{T} stored at of 5±3°C.

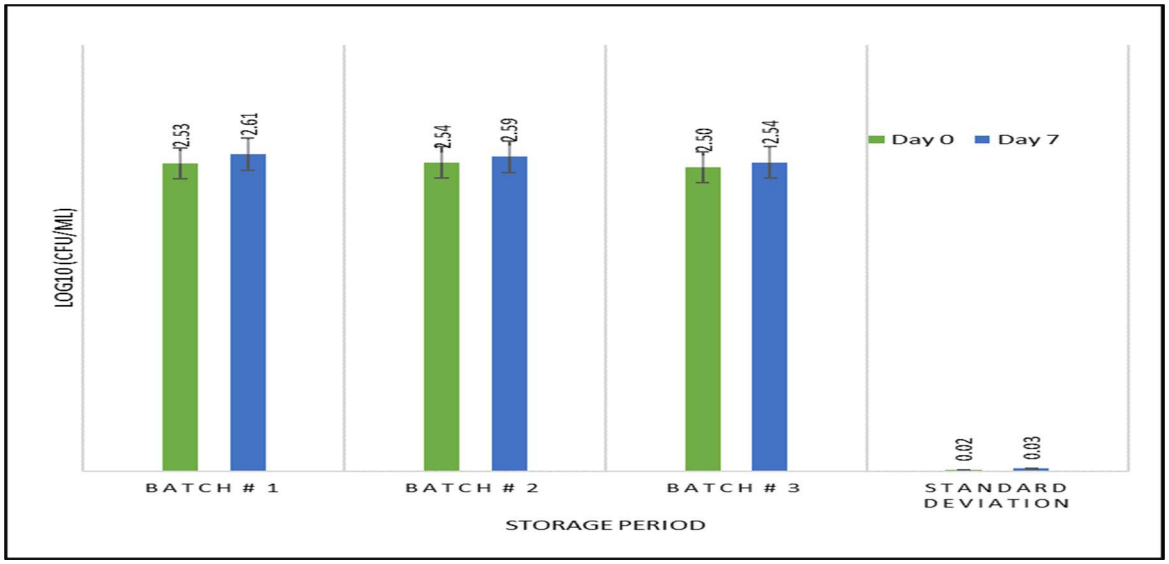


Figure 51. Viability study data of *S. enterica* subspp. Arizonae MTCC 660^{T} quantitative reference material stored at room temperature (31±5°C).

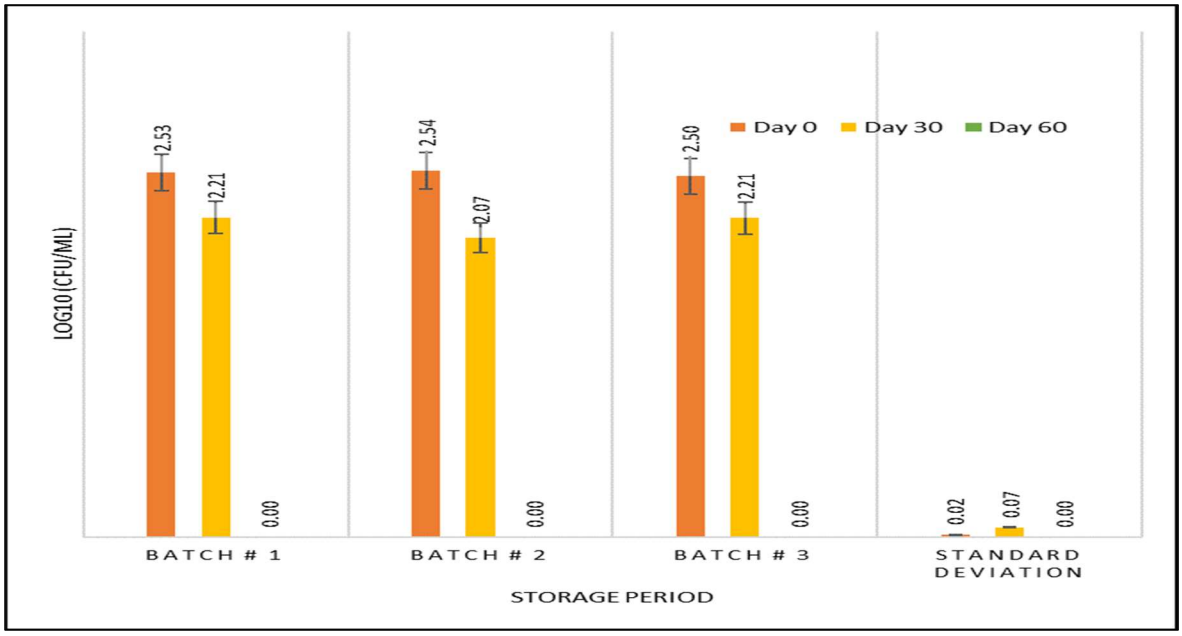


Figure 52. Viability study data of *S. enterica* subspp. Arizonae MTCC 660^{T} quantitative reference material stored at refrigeration conditions (5±3°C).

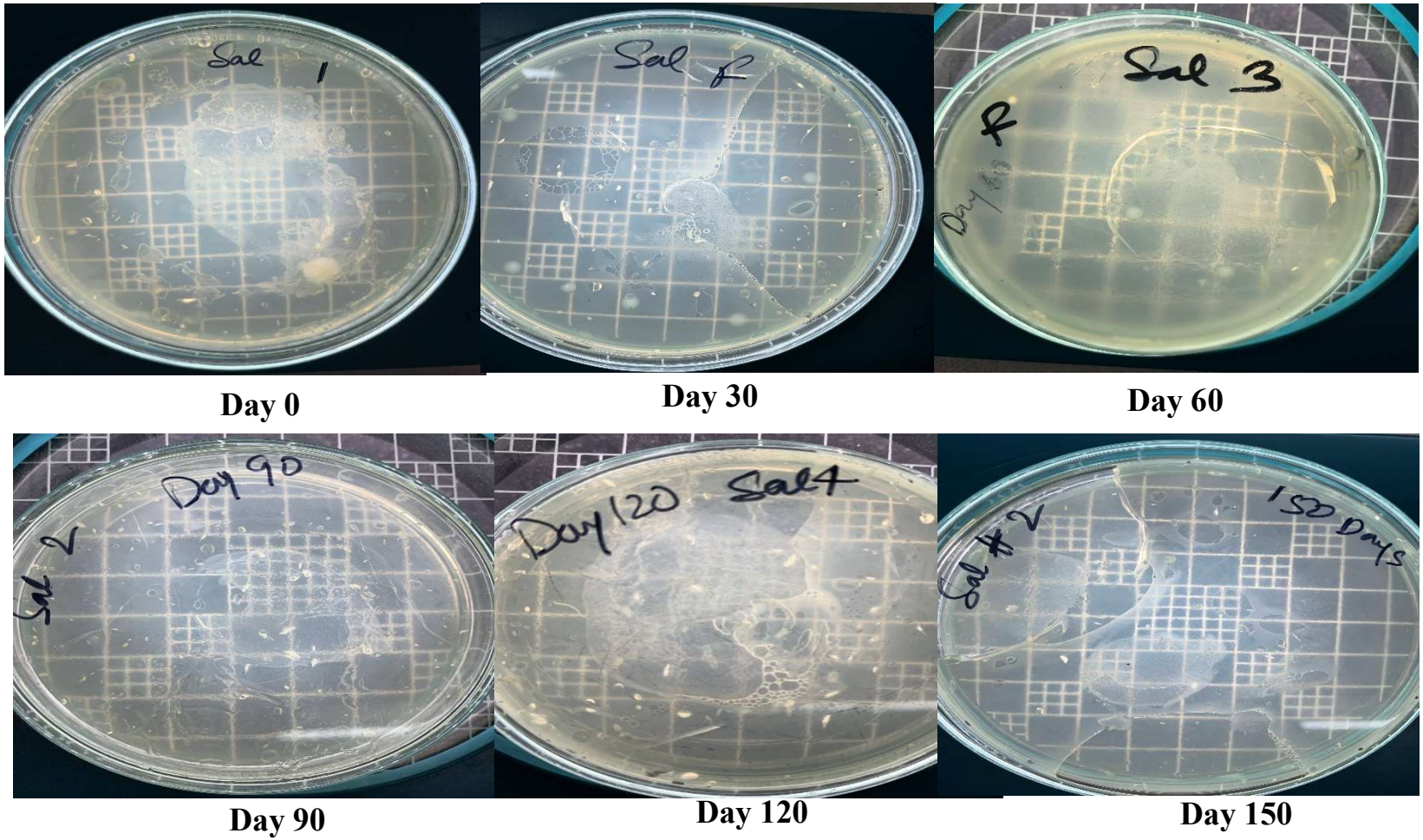


Figure 53. Photograph of viability study conducted for produced quantitative reference material of *S. enterica* subspp. Arizonae MTCC 660^{T} stored at -18±3°C.

Table 17: Viability study data converted to logarithmic value for 180 Days and One-way ANOVA analysis result for quantitative reference material of *S. enterica* subspp. Arizonae MTCC 660^{T} at -18±3°C till 120 Days

	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180
Batch # 1	2.53	2.64	2.27	2.29	2.31	1.65	0.00
Batch # 2	2.54	2.61	2.26	2.30	2.28	1.10	0.00
Batch # 3	2.50	2.61	2.42	2.29	2.31	1.46	0.00
Average Count	2.52	2.62	2.32	2.29	2.30	1.40	0.00
Standard Deviation (SD)	0.02	0.01	0.07	0.01	0.01	0.23	0.00
Overall SD			0.14*			- -	- -

Groups	*Count*	*Sum*	*Average*	*Variance*
Batch # 1	5	12.03512438	2.407024875	0.027746858
Batch # 2	5	11.96472173	2.392944346	0.028586607
Batch # 3	5	12.0914119	2.41828238	0.020319029

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001611681	2	0.000805841	0.031538722	0.969033477	3.885293835
Within Groups	0.306609977	12	0.025550831			
Total	0.308221658	14				

* SD calculated till 120 days as it was >0.35 Log_{10} at 150 days

The second objective was to: Develop quantitative RM with food matrix (skimmed milk powder) and compare viability with direct inoculation and in tube format.

6.8 Development of proficiency testing sample by inclusion of food matrix in developed quantitative reference materials

As all the commercially available SMP are not sterile, and to sterilize SMP by autoclaving, one must prepare a 10% solution of SMP in distilled water, which represents reconstituted milk. A similar study involving application testing of cryopreserved *Staphylococcus aureus* microspheres was directly added in milk procured from the market to study the recovery rate in the food matrix (242). The direct inclusion of SMP in the produced quantitative RM resulted in bacterial contamination. Hence, autoclaved SMP solution (10%) prepared in distilled water was used for addition in the quantitative RM, followed by assessing viability or recovery (Figure 54). Tables 18 and 19 detail the results of the viability study or recovery of both the target microorganisms after adding SMP as a food matrix in quantitative RM prepared in the first objective. The counts observed were converted to Log_{10} values, and the SD was calculated. SD observed for *E. coli* and *S. enterica* were 0.07 and 0.11, respectively, which is lower than the acceptable SD of 0.35 Log_{10} (65). The expected range calculated for quantitative RM of *E. coli* was 2.06 – 2.76, all the readings observed in Table 18 was observed to be within the assigned range of quantitative RM calculated in 6.7.1. The expected range calculated for quantitative RM of *S. enterica* was 1.96 – 2.66, all the readings observed in Table 19 was observed to be within the assigned range of quantitative RM calculated in 6.7.2.

The viability study data observed for quantitative RM of *S. enterica* MTCC 660^T and *E. coli* MTCC 1610^T with the inclusion of SMP as food matrix does not have any significant impact on the recovery of both the analytes. Hence with the produced quantitative RM, SMP can be included as a food matrix to develop PT samples.

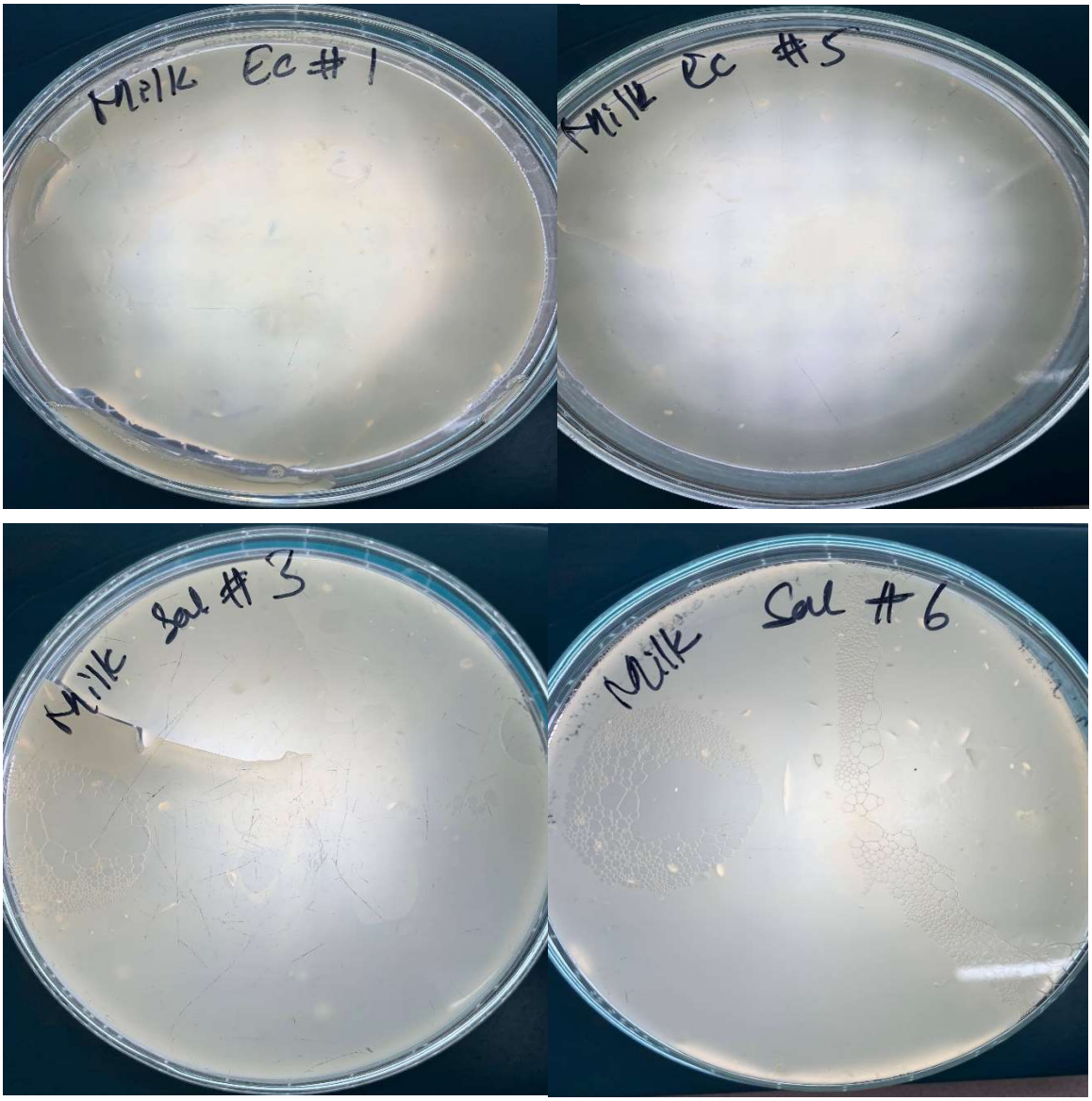


Figure 54. Photograph of recovery study conducted for produced quantitative RM of *E. coli* MTCC 1610^{T} and *S. enterica* subspp. Arizonae MTCC 660^{T} along with inclusion of 10% SMP.

Table 18: Viability study data for quantitative reference materials of *E. coli* MTCC 1610^T with inclusion of skimmed milk powder as food matrix

Sample Nos.	Observed (CFU)	Log_{10} (Observed)	z-score	O-E	sq(O-E)	sq(O-E)/E
1	237	2.37	-0.10	-0.04	0.00	0.00
2	249	2.40	-0.04	-0.01	0.00	0.00
3	192	2.28	-0.36	-0.13	0.02	0.01
4	216	2.33	-0.22	-0.08	0.01	0.00
5	306	2.49	0.22	0.08	0.01	0.00
6	231	2.36	-0.13	-0.05	0.00	0.00
7	258	2.41	0.00	0.00	0.00	0.00
8	188	2.27	-0.39	-0.14	0.02	0.01
9	174	2.24	-0.48	-0.17	0.03	0.01
10	210	2.32	-0.25	-0.09	0.01	0.00
Expected Result		**2.41 (2.06 – 2.76)**			Sum of variance (Chi -square calculated)	**0.04**
Standard Deviation		**0.07**				

Table 19: Viability study data for quantitative reference materials of *S. enterica* MTCC 660^T with inclusion of skimmed milk powder as food matrix

Sample Nos.	Observed (CFU)	Log_{10} (Observed)	z-score	O-E	sq(O-E)	sq(O-E)/E
1	264	2.42	0.32	0.11	0.01	0.01
2	282	2.45	0.40	0.14	0.02	0.01
3	288	2.46	0.43	0.15	0.02	0.01
4	309	2.49	0.51	0.18	0.03	0.01
5	162	2.21	-0.29	-0.10	0.01	0.00
6	189	2.28	-0.10	-0.03	0.00	0.00
7	315	2.50	0.54	0.19	0.04	0.02
8	222	2.35	0.10	0.04	0.00	0.00
9	222	2.35	0.10	0.04	0.00	0.00
10	174	2.24	-0.20	-0.07	0.00	0.00

Expected Result	**2.31 (1.96 – 2.66)**	Sum of variance (Chi -square calculated)	**0.06**
Standard Deviation	**0.11**		

6.9 Determination of homogeneity and stability of proficiency testing samples

Equations 5 and 6 were used to calculate the z-score and Chi-square, respectively, based on the viability study data obtained from Tables 25 and 26. These calculations aimed to determine the homogeneity and stability of the sample. The z-score observed for both studies fell well within the acceptable range of +2 and -2, and the observed result was within the narrower range of +1 and -1. The observed viability was compared with the expected value without the inclusion of SMP as a food matrix; the sum of variance or Chi-square values observed was 0.04 and 0.06 for *E. coli* and *S. enterica,* respectively. The tabulated Chi-square values at 9 degrees of freedom (n–1) at 0.05 α and 0.995 α are 16.92 and 1.73. The calculated value of Chi-square is < 0.05 α and < 0.995 α. Since our test statistics or calculated Chi-square value is less than the tabulated or critical Chi-square value, we fail to reject the null hypothesis, and we do not have sufficient evidence to say that the tested samples are non-homogeneous.

The third objective was the validation of developed quantitative RM tube with commercially available quantitative RM

6.10 Commercial quantitative reference material study

Ten samples of commercially available quantitative RM of *E. coli* and *S. enterica* were tested on TSA (Figure 55). The results obtained for commercially available quantitative RM were observed to be within the standard deviation of Log_{10} 0.35 and as per the acceptable range mentioned in the certificate of analysis. The assigned or certified value for *E. coli* commercial quantitative RM was 93 CFU, and the expected range was 47 – 190 CFU as per the certificate of analysis. Similarly, for *S. enterica* commercial quantitative RM, the expected range was 46 – 230 CFU with an assigned or expected mean value of 100 CFU as per the certificate of analysis. The observed value for each of the samples of commercial quantitative RM was within the expected range as per the certificate of analysis (Tables 20 and 21). The standard deviation calculated for the observed value was 0.21 <

0.35 (acceptable limit of standard deviation) for both the tested microorganisms. The shelf life was 2 years indicating better stability of the commercial quantitative RM.

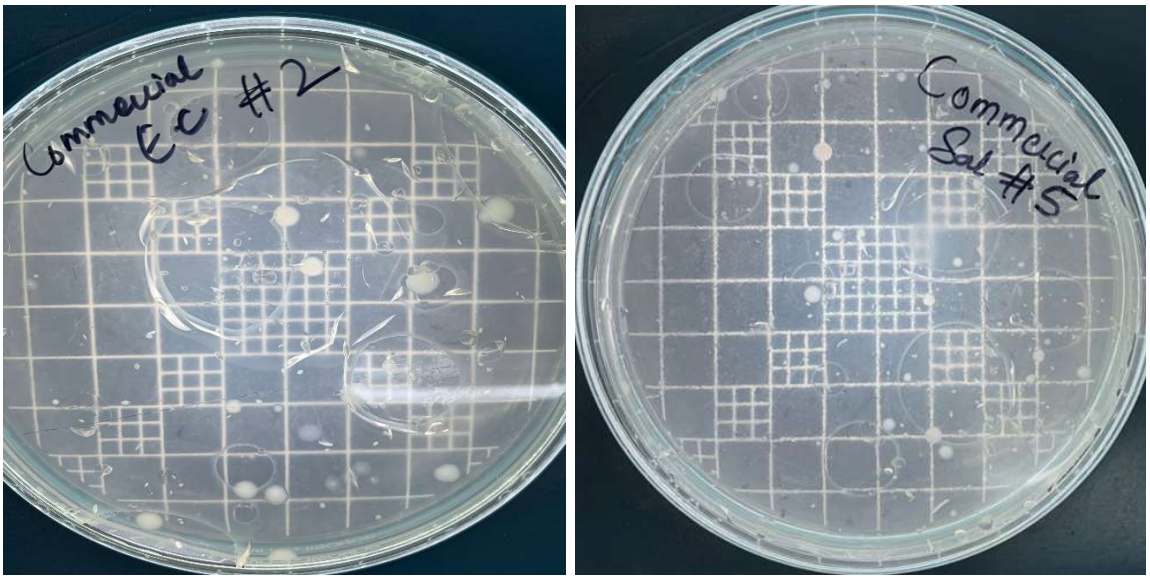


Figure 55. Photograph of commercial quantitative RM of *E. coli* WDCM 00013 and *S. enterica* Enteritidis WDCM 00030 tested using TSA at 37±1°C for 18 - 24 h.

Table 20: Viability study data for commercial quantitative reference materials of *Escherichia coli* WDCM 00013 VT000133

Sample Nos.	Observed (CFU)	Log_{10}(Observed)
1	68	1.83
2	108	2.03
3	206	2.31
4	142	2.15
5	189	2.28
6	154	2.19
7	67	1.83
8	104	2.02
9	150	2.18
10	49	1.69
Standard deviation calculated		**0.21**
Expected range as per certificate of analysis		**47 – 190 CFU**
Assigned value as per certificate of analysis		**93 CFU**

Table 21: Viability study data for commercial quantitative reference materials of *Salmonella enterica* subsp. *enterica* serovar Enteritidis WDCM 00030 VT000303

SAMPLE NOS.	OBSERVED (CFU)	LOG_{10}(OBSERVED)
1	103	2.01
2	54	1.73
3	56	1.75
4	208	2.32
5	48	1.68
6	118	2.07
7	120	2.08
8	49	1.69
9	106	2.03
10	66	1.82
Standard deviation calculated		**0.21**
Expected range as per certificate of analysis		**46 – 230 CFU**
Assigned value as per certificate of analysis		**100 CFU**

6.11 External validation of developed quantitative reference materials

The results obtained for the produced quantitative RM from five different institutes were observed to be well within the assigned value range based on the in-house viability study (Tables 22 and 23). z -score of each of the reported readings by the institutes were well within the acceptable range of +2 to -2 indicating the acceptability of the produced quantitative RM. The standard deviation observed for the reported institute was 0.07 which is less than the acceptable standard deviation (SD = 0.35).

Table 22*:* Viability study data tested by institutes for produced quantitative reference materials of *E. coli* MTCC 1610^{T}

Sr. No.	Institute Code*	Result Reported (CFU)	Log_{10}(Result)	z-score
1	CL 1	357	2.55	0.44
2	CL 2	330	2.52	0.02
3	CL 3	370	2.57	0.48
4	AI 1	256	2.41	-0.30
5	AI 2	289	2.46	-0.12
			Average Mean	2.50
			Standard Deviation	0.07
			Expected Mean	2.40

*****Table 6 contains the details (name and type) of the institutes

Table 23: Viability study data tested by institutes for produced quantitative reference materials of *S. enterica* MTCC 660^{T}

Sr. No.	Institute Code	Result Reported (CFU)	Log_{10}(Result)	z-score
1	CL 1	235	2.37	-0.11
2	CL 2	220	2.34	-0.23
3	CL 3	310	2.49	0.23
4	AI 1	310	2.49	0.19
5	AI 2	274	2.44	0.03
			Average Mean	2.43
			Standard Deviation	0.07
			Expected Mean	2.41

In assessing the performance of both commercial and developed quantitative RM, we observed that the standard deviation for the latter (0.07) was lower than that for the former (0.21). This indicates reduced variation in the developed quantitative RM. However, it is important to note that the long-term stability achieved by the commercial quantitative RM over two years is superior and more desirable compared to the four-month stability achieved by the developed quantitative RM. Both commercial and developed RM fall within the expected range and exhibit an acceptable standard deviation.

CHAPTER 7

SUMMARY AND CONCLUSION

7.0 Summary & Conclusions

Quantitative reference materials (RM) play a significant role in ensuring the quality of data produced in testing laboratories. The amount of research available in the Scopus database for microbiological certified value reference material is ~1.3% compared to ~ 87.5% for chemical and related certified reference material. This indicates that sufficient studies are not available on the development of quantitative or certified value reference materials in microbiology. Quantitative microbiological RM are mostly imported from the USA or the EU to India. This is true even for other countries where quantitative microbiological RM are not produced. Quantitative microbiological RM are currently procured by developing countries via importing them from the few meager international institutes situated only in developed economies like the EU, USA, or the UK. In this context, developing countries should be encouraged and supported to develop their own quantitative RM indigenously to overcome this lacuna and convert this challenge into a great opportunity for creating self-sufficiency as well as to improve their economy. There is also a great scope for improvement in the application of quantitative RM, like capacity building and training. There are different formats available for RM in microbiology, like quantitative or qualitative viable cells, combined in different food matrices like milk powders, cheese, etc., and nuclear materials, toxins produced by pathogenic food bacteria.

One of the main purposes of this study, hence, was to bridge this gap by developing quantitative RM of *E. coli* and *S. enterica*, the two most important foodborne micro-organisms involved in the majority of food safety recalls across the globe. The initial step covered in this study is the assessment of nutritive and protective agents, along with the application of preservation techniques to develop quantitative RM. The essential points to consider in the development of microbiological RM are homogeneity and stability at different temperatures, involving storage & transportation. Different media, methods of analysis, interfering agents, and complex food matrices can also impact the recovery of microorganisms in reference material. For certified reference materials, traceability to the SI unit is essential when calculating certified values. Additionally, incorporating

uncertainty measurements is crucial. Since quantitative microbiology RM are currently imported, the cost of procurement is not easily affordable. Hence affordable assigned value RM in this field will increase its utilization in routine testing. RM with certified value can also be used for the capacity building of academic students in microbiology.

For the selection of nutritive agents, different protein hydrolysates were explored. Protein hydrolysates have wider applications, apart from their nutritional properties; they exhibit biological properties, including antioxidant, antimicrobial, cholesterol-reducing, hypoglycemic, and anticancer effects. Bioactive peptides from protein hydrolysates have gained significant attention, and several studies have explored their antimicrobial properties and as alternatives to peptones in microbiological media as a nitrogen source. In this study, we evaluated the nutritive properties of animal and plant-based protein hydrolysates for the creation of a secondary reference material for *E. coli* and *Salmonella*. The key findings were that FPH was the only hydrolysate that effectively maintained the long-term viability of *S. enterica*, whereas both SPH and FPH can act as nutritive agents and support the survival of *E. coli*. Inoculum grown on agar further aided the survival of *E. coli* and *S. enterica*, resulting in increased recovery and better viability in FPH. Additionally, during the RSM optimization study, we observed that 5% FPH from *Stolephorus indicus* led to better recovery and prolonged survival of *S. enterica* and *E. coli* under refrigeration conditions, but not under freezing conditions. Further, casein hydrolysate and SPH can be utilized to isolate bioactive peptides and as food additives for *Salmonella* control in food and food-contact packaging materials. Protein hydrolysates did not exhibit any cryoprotection at freezing conditions. The freezing condition is the most suitable temperature to keep the microbial cells dormant, which has a major influence on standard deviation which is the most important requirement of assigned value RM. Hence, protective agents like trehalose, glycerol, glucose, and bovine serum albumin (BSA) were explored for inclusion in the formulation to create a secondary RM.

The presence of trehalose, glucose, and sucrose, which act as growth-promoting factors, has been shown to have a positive impact on the survival of microorganisms.

Therefore, these protective agents (trehalose, glucose, and glycerol) were individually assessed on *E. coli* and *S. enterica* for their survival at 5, 10, 15, 20 and 25% concentrations whereas BSA was studied at 5 and 10% concentrations at refrigeration and freezing conditions for 30 days. This was followed by an RSM optimization study to evaluate any synergistic or antagonistic effects among the protective agents. Key findings revealed that glycerol and glucose have antagonistic effects. For optimal survival at freezing temperatures, glycerol proved more effective than glucose, necessitating a higher concentration of glycerol in the formulation. RSM is an effective tool for optimizing experimental runs and establishing relationships between variables. However, it may lead to a higher standard deviation (SD) when microbial viability is used as a response. FPH (5%), glycerol (15%), trehalose (10%), glucose (1%), and BSA (2.5%) were used in the matrix formulation of quantitative RM. The encapsulation step was used as a preservation technique, wherein 3% gelatin and 2% CMC were introduced to reduce water activity and make the final product shelf stable. With the inclusion of protective agents and an encapsulation step, freezing conditions (-18±3°C) were identified as the most suitable storage temperature. Thus, quantitative RM for microbiology testing was developed using protein hydrolysate, cryoprotective agents, and an encapsulation technique. The produced quantitative RM of *E. coli* and *S. enterica* demonstrated viability till 150 days at -18±3°C, compared to their viability till 30 days at 5±3°C. This suggests that encapsulation is crucial for extending the shelf life of the quantitative RM when stored at freezing conditions. The study also highlighted the importance of low water activity, freezing temperature (-18±3°C), and the inclusion of cryoprotectants in the matrix formulation for the quantitative reference material to achieve improved shelf stability. All three batches of *S. enterica* were found to be homogeneous and stable for up to 120 days, exhibiting an SD of 0.14 Log_{10}, well within the limit of 0.35 Log_{10}. The three batches of *E. coli* were found to have homogeneity and viability till 150 days with 0.33 SD and homogeneity and stability till 120 days with SD of 0.17 Log_{10}. The sudden loss in viability observed at 180 days suggests that other protective agents and further lower temperatures could be explored, along with

the identification of formulation robustness with the inclusion of other strains of the target micro-organisms.

The comparative assessment of developed and commercially available quantitative RM was conducted using SD. The SD observed for both developed and commercial quantitative RM was < 0.35 Log_{10}. The shelf-life range of commercially available quantitative RM was two years, against 120 days of the in-house developed quantitative RM. The developed quantitative RM has potential applications in microbiological analysis or testing methods. It can demonstrate the performance of media, reagents, and sterile consumables, and shows promise for a standalone application in growth promotion tests for microbiological media qualification in food testing laboratories. To extend the reach of the created assigned value reference materials, there is a strong need to increase their shelf life. This can be achieved by further improving the encapsulation process and reducing the overall water activity of the product.

The developed quantitative RM were used to formulate proficiency testing (PT) samples by including food matrix in the formulation of quantitative RM. The application of PT samples in food testing laboratories are for method validation, method verification, and training of analysts as a part of competence or skill development. Based on the data available on the survival of pathogens in different food matrices, it can be concluded that several factors are interlinked or interdependent and responsible for aiding in the survival of these foodborne pathogens in food matrices. In this study, we studied the effect of the inclusion of SMP as a food matrix in the developed quantitative RM for the creation of PT samples. The direct inclusion of SMP in developed quantitative RM resulted in bacterial contamination; hence, 10% SMP (autoclaved) was used for the study. The effect of the addition of SMP on the survival or viability of the quantitative RM were studied along with assessment of homogeneity using z-score and Chi-square. z-score observed for the quantitative RM of *E. coli* and *S. enterica* were between ± 1 as against the value of ± 2. Chi-square test resulted in acceptance of the null hypothesis, demonstrating its homogeneous nature and thus can be used for its said applications.

CHAPTER 8

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APPENDIX

Appendix 1: Composition of medias and diluents used

Table 1: Composition of TSB

Ingredients	Quantity (g/L)
Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dextrose	2.50
Dibasic potassium phosphate	2.50
Distilled Water	1.0 L
Final pH (at 25°C)	7.3±0.2

Table 2: Composition of TSA

Ingredients	Quantity (g/L)
Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dextrose	2.50
Dibasic potassium phosphate	2.50
Agar	15.0
Distilled Water	1.0 L
Final pH (at 25°C)	7.4±0.2

Table 3: Composition of saline

Ingredients	Quantity (g/100 mL)
Sodium Chloride (NaCl)	0.85
Distilled Water	100.0 mL
Final pH (at 25°C)	6.2±0.5

Table 4: Composition of MacConkey agar

Ingredients	Quantity (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.070
Agar	15.0
Distilled Water	1.0 L
Final pH (at 25°C)	7.5±0.2

Table 5: Composition of XLD agar

Ingredients	Quantity (g/L)
Yeast extract	3.0
L-Lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.50
Sodium thiosulphate	6.80

Ferric ammonium citrate	0.80
Phenol red	0.080
Agar	15.0
Distilled Water	1.0 L
Final pH (at 25°C)	7.4±0.2

Table 6: Composition of nutrient broth

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Distilled Water	1.0 L
Final pH (at 25°C)	7.4±0.2

Table 7: Composition of nutrient agar

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Distilled Water	1.0 L
Final pH (at 25°C)	7.4±0.2

Appendix 2: Raw data from RSM software for protein hydrolysates study of *S. enterica*

ANOVA for Quadratic model

Response 1: Day 0

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.93	9	0.8811	291.56	< 0.0001	significant
A-CH	0.9384	1	0.9384	310.52	< 0.0001	
B-SPH	6.86	1	6.86	2271.07	< 0.0001	
C-FPH	0.0300	1	0.0300	9.93	0.0161	
AB	0.0441	1	0.0441	14.59	0.0065	
AC	0.0225	1	0.0225	7.45	0.0294	
BC	0.0000	1	0.0000	0.0083	0.9301	
A^2	0.0150	1	0.0150	4.97	0.0610	
B^2	0.0138	1	0.0138	4.57	0.0700	
C^2	0.0022	1	0.0022	0.7211	0.4239	
Residual	0.0212	7	0.0030			
Lack of Fit	0.0175	3	0.0058	6.33	0.0533	not significant
Pure Error	0.0037	4	0.0009			
Cor Total	7.95	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 291.56 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 6.33 implies there is a 5.33% chance that a Lack of Fit F-value this large could occur due to noise. Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling.

Fit Statistics

Std. Dev.	0.0550	**R²**	0.9973
Mean	1.57	**Adjusted R²**	0.9939
C.V. %	3.50	**Predicted R²**	0.9641
		Adeq Precision	60.1829

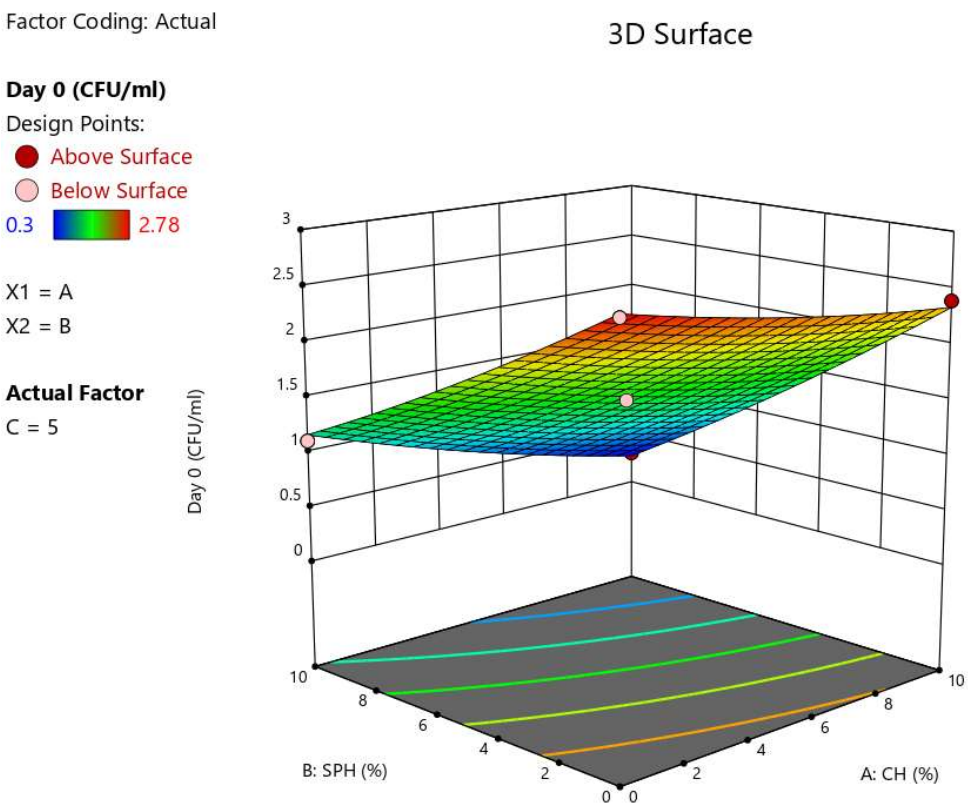
The **Predicted R²** of 0.9641 is in reasonable agreement with the **Adjusted R²** of 0.9939; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 60.183 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	1.18	1.24	-0.0563	0.750	-2.046	-2.989	1.256[1]	-5.177[1]	8
2	1.57	1.53	0.0420	0.200	0.854	0.836	0.018	0.418	13
3	2.78	2.81	-0.0287	0.750	-1.046	-1.054	0.328	-1.826	1
4	2.39	2.33	0.0563	0.750	2.046	2.989	1.256[1]	5.177[1]	2
5	1.11	1.17	-0.0563	0.750	-2.046	-2.989	1.256[1]	-5.177[1]	3
6	1.53	1.53	0.0020	0.200	0.041	0.038	0.000	0.019	17
7	1.18	1.21	-0.0288	0.750	-1.046	-1.054	0.328	-1.826	6
8	1.49	1.53	-0.0380	0.200	-0.773	-0.748	0.015	-0.374	16
9	1.54	1.53	0.0120	0.200	0.244	0.227	0.001	0.113	15
10	0.70	0.70	0.0000	0.750	0.000	0.000	0.000	0.000	10
11	1.51	1.53	-0.0180	0.200	-0.366	-0.342	0.003	-0.171	14
12	2.52	2.55	-0.0275	0.750	-1.000	-1.001	0.300	-1.733	9
13	1.80	1.77	0.0288	0.750	1.046	1.054	0.328	1.826	7
14	2.43	2.43	0.0000	0.750	0.000	0.000	0.000	0.000	11
15	2.10	2.04	0.0562	0.750	2.046	2.989	1.256[1]	5.177[1]	5
16	0.60	0.57	0.0275	0.750	1.000	1.001	0.300	1.733	12
17	0.30	0.27	0.0287	0.750	1.046	1.054	0.328	1.826	4

[1] Exceeds limits.



ANOVA for Linear model

Response 2: Day 7

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	11.62	3	3.87	403.13	< 0.0001	significant
A-CH	2.74	1	2.74	284.91	< 0.0001	
B-SPH	8.88	1	8.88	924.42	< 0.0001	
C-FPH	0.0006	1	0.0006	0.0637	0.8046	
Residual	0.1249	13	0.0096			
Lack of Fit	0.1072	9	0.0119	2.69	0.1769	not significant
Pure Error	0.0177	4	0.0044			
Cor Total	11.75	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 403.13 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 2.69 implies the Lack of Fit is not significant relative to the pure error. There is a 17.69% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.0980	**R²**	0.9894
Mean	1.42	**Adjusted R²**	0.9869
C.V. %	6.89	**Predicted R²**	0.9792
		Adeq Precision	68.9270

The **Predicted R²** of 0.9792 is in reasonable agreement with the **Adjusted R²** of 0.9869; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 68.927 indicates an adequate signal. This model can be used to navigate the design space.

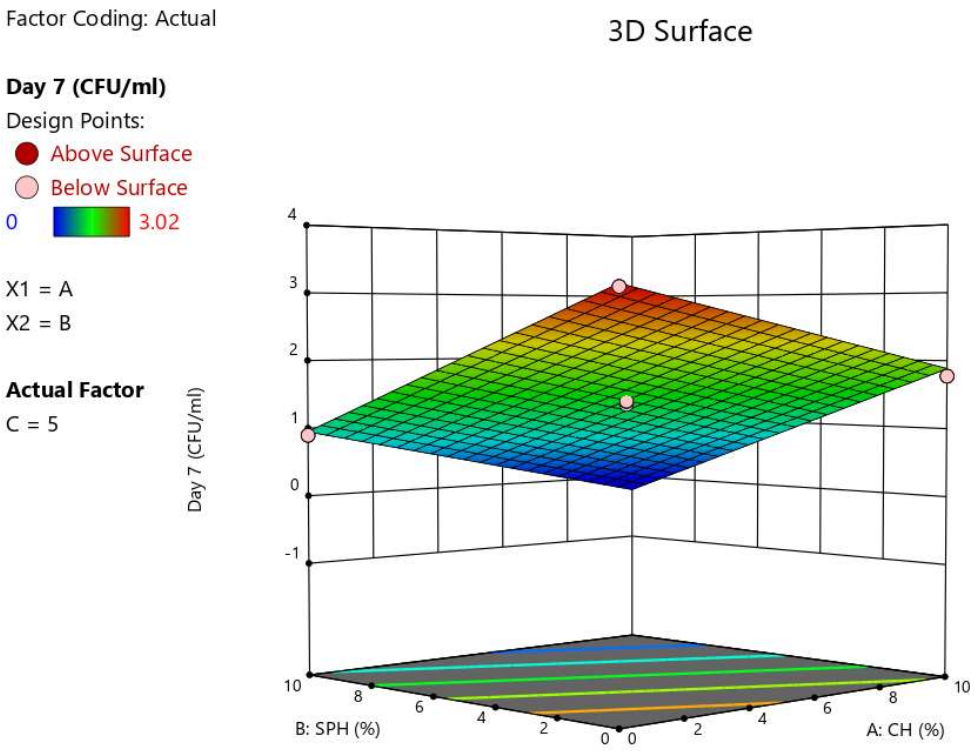
Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.78	0.8280	-0.0480	0.309	-0.589	-0.574	0.039	-0.384	8
2	1.38	1.42	-0.0418	0.059	-0.439	-0.425	0.003	-0.106	13
3	3.02	3.06	-0.0405	0.309	-0.497	-0.482	0.028	-0.322	1
4	1.78	1.89	-0.1105	0.309	-1.356	-1.406	0.205	-0.940	2
5	0.90	0.9530	-0.0530	0.309	-0.651	-0.635	0.047	-0.425	3
6	1.53	1.42	0.1082	0.059	1.138	1.152	0.020	0.288	17
7	0.78	0.8455	-0.0655	0.309	-0.804	-0.792	0.072	-0.530	6
8	1.41	1.42	-0.0118	0.059	-0.124	-0.119	0.000	-0.030	16
9	1.40	1.42	-0.0218	0.059	-0.229	-0.220	0.001	-0.055	15
10	0.30	0.3768	-0.0768	0.309	-0.942	-0.938	0.099	-0.627	10

11	1.36	1.42	-0.0618	0.059	-0.649	-0.634	0.007	-0.159	14
12	2.54	2.48	0.0557	0.309	0.684	0.669	0.052	0.447	9
13	1.99	2.00	-0.0080	0.309	-0.098	-0.095	0.001	-0.063	7
14	2.59	2.47	0.1232	0.309	1.512	1.600	0.255	1.070	11
15	2.11	2.02	0.0945	0.309	1.159	1.176	0.150	0.786	5
16	0.30	0.3593	-0.0593	0.309	-0.727	-0.713	0.059	-0.477	12
17	0.00	-0.2170	0.2170	0.309	2.662	3.794[1]	0.792	2.536[2]	4

[1] Observation with |External Stud. Residuals| > 3.72

[2] Exceeds limits.



ANOVA for Linear model

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value

Model	4.84	3	1.61	345.50	< 0.0001	significant
A-CH	1.08	1	1.08	231.39	< 0.0001	
B-SPH	3.59	1	3.59	769.08	< 0.0001	
C-FPH	0.1682	1	0.1682	36.02	< 0.0001	
Residual	0.0607	13	0.0047			
Lack of Fit	0.0458	9	0.0051	1.36	0.4082	not significant
Pure Error	0.0149	4	0.0037			
Cor Total	4.90	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 345.50 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.36 implies the Lack of Fit is not significant relative to the pure error. There is a 40.82% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

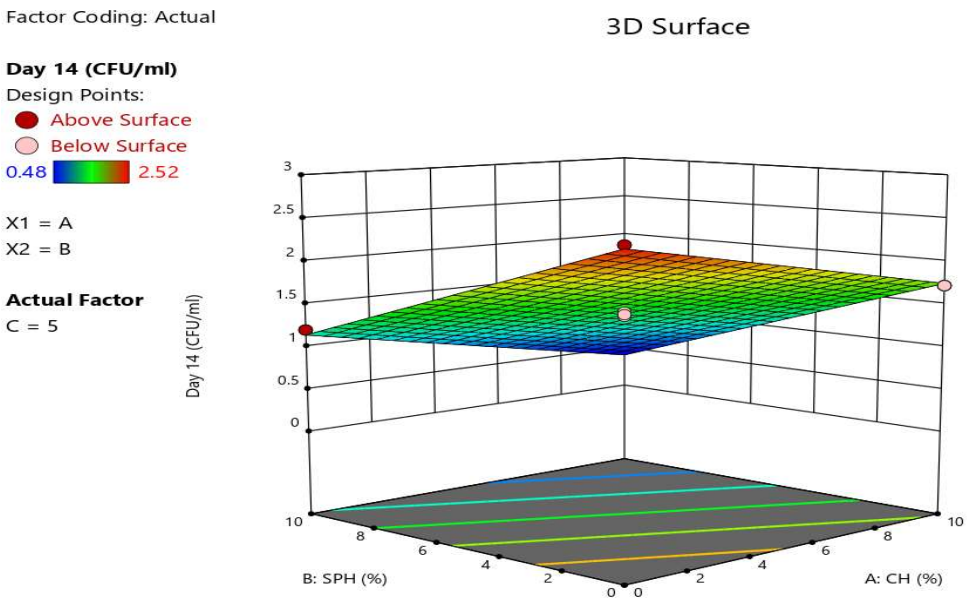
Std. Dev.	0.0683	**R^2**	0.9876
Mean	1.44	**Adjusted R^2**	0.9848
C.V. %	4.74	**Predicted R^2**	0.9773
		Adeq Precision	62.6007

The **Predicted R^2** of 0.9773 is in reasonable agreement with the **Adjusted R^2** of 0.9848; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 62.601 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	1.18	1.22	-0.0404	0.309	-0.712	-0.698	0.057	-0.466	8
2	1.49	1.44	0.0471	0.059	0.710	0.696	0.008	0.174	13
3	2.52	2.48	0.0396	0.309	0.696	0.682	0.054	0.456	1
4	1.72	1.75	-0.0254	0.309	-0.448	-0.434	0.022	-0.290	2
5	1.20	1.14	0.0596	0.309	1.048	1.053	0.123	0.704	3
6	1.38	1.44	-0.0629	0.059	-0.949	-0.946	0.014	-0.236	17
7	0.90	0.9304	-0.0304	0.309	-0.536	-0.521	0.032	-0.348	6
8	1.49	1.44	0.0471	0.059	0.710	0.696	0.008	0.174	16
9	1.41	1.44	-0.0329	0.059	-0.497	-0.482	0.004	-0.121	15
10	0.70	0.6279	0.0721	0.309	1.268	1.302	0.180	0.870	10
11	1.36	1.44	-0.0829	0.059	-1.251	-1.282	0.024	-0.320	14
12	1.99	1.97	0.0221	0.309	0.388	0.375	0.017	0.251	9
13	1.93	1.96	-0.0254	0.309	-0.448	-0.434	0.022	-0.290	7
14	2.36	2.26	0.1021	0.309	1.796	1.991	0.361	1.331	11
15	1.57	1.67	-0.0954	0.309	-1.680	-1.824	0.315	-1.219	5
16	0.85	0.9179	-0.0679	0.309	-1.196	-1.218	0.160	-0.814	12
17	0.48	0.4054	0.0746	0.309	1.312	1.354	0.192	0.905	4



ANOVA for Linear model

Response 4: Day 21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12.19	3	4.06	210.35	< 0.0001	significant
A-CH	1.02	1	1.02	52.92	< 0.0001	
B-SPH	11.04	1	11.04	571.66	< 0.0001	
C-FPH	0.1250	1	0.1250	6.47	0.0245	
Residual	0.2512	13	0.0193			
Lack of Fit	0.1211	9	0.0135	0.4137	0.8751	not significant
Pure Error	0.1301	4	0.0325			
Cor Total	12.44	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 210.35 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.41 implies the Lack of Fit is not significant relative to the pure error. There is a 87.51% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

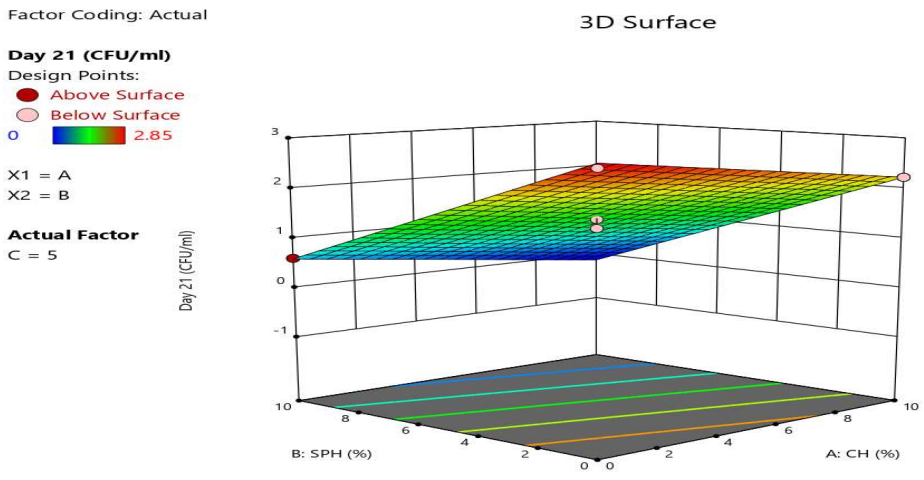
Std. Dev.	0.1390	**R^2**	0.9798
Mean	1.40	**Adjusted R^2**	0.9752
C.V. %	9.90	**Predicted R^2**	0.9681
		Adeq Precision	45.4579

The **Predicted R^2** of 0.9681 is in reasonable agreement with the **Adjusted R^2** of 0.9752; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 45.458 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	1.18	1.17	0.0078	0.309	0.067	0.065	0.001	0.043	8
2	1.38	1.40	-0.0247	0.059	-0.183	-0.176	0.001	-0.044	13
3	2.85	2.94	-0.0872	0.309	-0.755	-0.741	0.064	-0.496	1
4	2.22	2.22	-0.0022	0.309	-0.019	-0.018	0.000	-0.012	2
5	0.60	0.5872	0.0128	0.309	0.111	0.106	0.001	0.071	3
6	1.60	1.40	0.1953	0.059	1.448	1.519	0.033	0.380	17
7	0.90	0.9222	-0.0222	0.309	-0.192	-0.185	0.004	-0.124	6
8	1.20	1.40	-0.2047	0.059	-1.518	-1.608	0.036	-0.402	16
9	1.20	1.40	-0.2047	0.059	-1.518	-1.608	0.036	-0.402	15
10	0.00	0.1047	-0.1047	0.309	-0.906	-0.899	0.092	-0.601	10
11	1.51	1.40	0.1053	0.059	0.781	0.768	0.010	0.192	14
12	2.63	2.45	0.1753	0.309	1.517	1.606	0.257	1.074	9
13	2.08	1.89	0.1928	0.309	1.668	1.808	0.311	1.209	7
14	2.60	2.70	-0.1047	0.309	-0.906	-0.899	0.092	-0.601	11
15	1.63	1.64	-0.0072	0.309	-0.062	-0.060	0.000	-0.040	5
16	0.30	0.3547	-0.0547	0.309	-0.473	-0.459	0.025	-0.307	12
17	0.00	-0.1278	0.1278	0.309	1.106	1.116	0.137	0.746	4



ANOVA for Quadratic model

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	13.31	9	1.48	403.15	< 0.0001	significant
A-CH	0.7938	1	0.7938	216.46	< 0.0001	
B-SPH	10.76	1	10.76	2935.47	< 0.0001	
C-FPH	1.07	1	1.07	290.63	< 0.0001	
AB	0.1056	1	0.1056	28.80	0.0010	
AC	0.0650	1	0.0650	17.73	0.0040	
BC	0.2756	1	0.2756	75.16	< 0.0001	
A^2	0.0070	1	0.0070	1.91	0.2098	
B^2	0.1672	1	0.1672	45.58	0.0003	
C^2	0.0550	1	0.0550	14.99	0.0061	
Residual	0.0257	7	0.0037			
Lack of Fit	0.0157	3	0.0052	2.12	0.2408	not significant
Pure Error	0.0099	4	0.0025			
Cor Total	13.33	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 403.15 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 2.12 implies the Lack of Fit is not significant relative to the pure error. There is a 24.08% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	**0.0606**	R^2	**0.9981**
Mean	1.32	**Adjusted R^2**	0.9956

C.V. %	4.58	**Predicted R²**	0.9799
		Adeq Precision	70.0828

The **Predicted R²** of 0.9799 is in reasonable agreement with the **Adjusted R²** of 0.9956; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 70.083 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	1.20	1.19	0.0100	0.750	0.330	0.308	0.033	0.534	8
2	1.28	1.19	0.0860	0.200	1.588	1.838	0.063	0.919	13
3	3.03	2.99	0.0400	0.750	1.321	1.412	0.524	2.445[(1)]	1
4	2.08	2.04	0.0450	0.750	1.486	1.663	0.663	2.881[(1)]	2
5	0.3000	0.3450	-0.0450	0.750	-1.486	-1.663	0.663	-2.881[(1)]	3
6	1.18	1.19	-0.0140	0.200	-0.258	-0.240	0.002	-0.120	17
7	0.7000	0.7150	-0.0150	0.750	-0.495	-0.467	0.074	-0.809	6
8	1.15	1.19	-0.0440	0.200	-0.812	-0.790	0.016	-0.395	16
9	1.18	1.19	-0.0140	0.200	-0.258	-0.240	0.002	-0.120	15
10	0.3000	0.2450	0.0550	0.750	1.816	2.313	0.990	4.006[(1)]	10
11	1.18	1.19	-0.0140	0.200	-0.258	-0.240	0.002	-0.120	14
12	2.01	2.04	-0.0300	0.750	-0.991	-0.989	0.295	-1.714	9
13	2.09	2.07	0.0150	0.750	0.495	0.467	0.074	0.809	7
14	3.24	3.30	-0.0550	0.750	-1.816	-2.313	0.990	-4.006[(1)]	11
15	1.08	1.09	-0.0100	0.750	-0.330	-0.308	0.033	-0.534	5
16	0.4800	0.4500	0.0300	0.750	0.991	0.989	0.295	1.714	12
17	0.0000	0.0400	-0.0400	0.750	-1.321	-1.412	0.524	-2.445[(1)]	4

[(1)] Exceeds limits.

Factor Coding: Actual

Day 28 (CFU/ml)

Design Points:

Above Surface

Below Surface

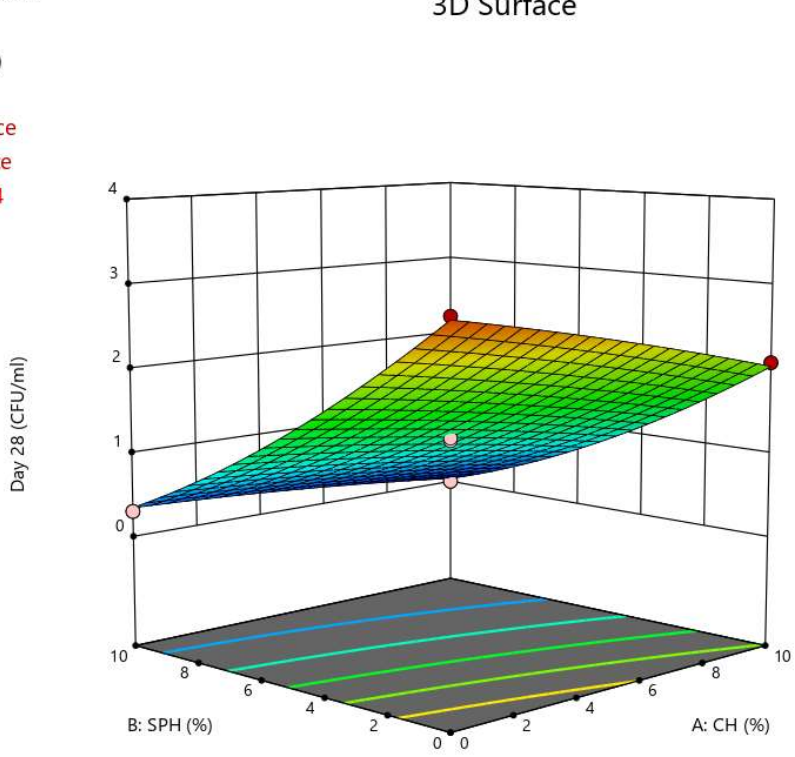
0 3.24

X1 = A

X2 = B

Actual Factor

C = 5



Appendix 3*:* Raw data from RSM software for protein hydrolysates study of *E. coli*

Build Information

File Version	22.0.0.1		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	17
Design Model	Quadratic	**Blocks**	No Blocks
Build Time (ms)	1.0000		

Factors

Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	CH	Percentage	Numeric	Continuous	0.0000	10.00	-1 ↔ 0.00	+1 ↔ 10.00	5.00	3.54
B	SPH	Percentage	Numeric	Continuous	0.0000	10.00	-1 ↔ 0.00	+1 ↔ 10.00	5.00	3.54
C	FPH	Percentage	Numeric	Continuous	0.0000	10.00	-1 ↔ 0.00	+1 ↔ 10.00	5.00	3.54

Fit Summary

Response 1: Day 0

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.2598**	**0.9125**	**0.8636**	**Suggested**
2FI	0.2125	0.3084	0.9260	0.8141	
Quadratic	0.9371	0.1443	0.9000	0.4851	
Cubic	0.1443		0.9487		**Aliased**

Sequential Model Sum of Squares [Type I]

Response 1: Day 0

Source	Sum of Squares	df	Mean Square	F-value	p-value

Mean vs Total	43.17	1	43.17			
Linear vs Mean	**8.06**	**3**	**2.69**	**56.62**	**< 0.0001**	**Suggested**
2FI vs Linear	0.2157	3	0.0719	1.79	0.2125	
Quadratic vs 2FI	0.0217	3	0.0072	0.1334	0.9371	
Cubic vs Quadratic	0.2685	3	0.0895	3.22	0.1443	Aliased
Residual	0.1113	4	0.0278			
Total	51.85	17	3.05			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

ANOVA for Linear model

Response 1: Day 0

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.06	3	2.69	56.62	< 0.0001	significant
A-CH	1.19	1	1.19	25.14	0.0002	
B-SPH	6.81	1	6.81	143.39	< 0.0001	
C-FPH	0.0630	1	0.0630	1.33	0.2701	
Residual	0.6172	13	0.0475			
Lack of Fit	0.5059	9	0.0562	2.02	0.2598	not significant
Pure Error	0.1113	4	0.0278			
Cor Total	8.68	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 56.62 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 2.02 implies the Lack of Fit is not significant relative to the pure error. There is a 25.98% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.2179	**R²**	0.9289
Mean	1.59	**Adjusted R²**	0.9125
C.V. %	13.67	**Predicted R²**	0.8636
		Adeq Precision	24.7648

The **Predicted R²** of 0.8636 is in reasonable agreement with the **Adjusted R²** of 0.9125; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 24.765 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.59	1	0.0528	1.48	1.71	
A-CH	-0.3862	1	0.0770	-0.5527	-0.2198	1.0000
B-SPH	-0.9225	1	0.0770	-1.09	-0.7561	1.0000
C-FPH	-0.0888	1	0.0770	-0.2552	0.0777	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.3000	0.2848	0.0152	0.309	0.084	0.081	0.001	0.054	4
2	0.6000	0.5823	0.0177	0.309	0.098	0.094	0.001	0.063	12
3	1.81	1.59	0.2165	0.059	1.024	1.026	0.016	0.257	13
4	2.52	2.60	-0.0848	0.309	-0.468	-0.453	0.024	-0.303	9
5	0.7000	0.7598	-0.0598	0.309	-0.330	-0.318	0.012	-0.213	10
6	2.63	2.43	0.2027	0.309	1.119	1.131	0.140	0.756	11
7	1.80	1.89	-0.0910	0.309	-0.503	-0.488	0.028	-0.326	7
8	1.53	1.59	-0.0635	0.059	-0.301	-0.290	0.001	-0.072	17
9	1.18	1.06	0.1227	0.309	0.677	0.663	0.051	0.443	3
10	2.48	2.13	0.3502	0.309	1.933	2.201	0.418	1.471[(1)]	2
11	0.9000	1.12	-0.2185	0.309	-1.206	-1.230	0.163	-0.822	8
12	2.53	2.90	-0.3723	0.309	-2.055	-2.403	0.472	-1.606[(1)]	1
13	1.08	1.30	-0.2160	0.309	-1.193	-1.214	0.159	-0.812	6
14	2.34	2.07	0.2715	0.309	1.499	1.583	0.251	1.058	5
15	1.48	1.59	-0.1135	0.059	-0.537	-0.522	0.005	-0.130	14
16	1.45	1.59	-0.1435	0.059	-0.679	-0.664	0.007	-0.166	15
17	1.76	1.59	0.1665	0.059	0.788	0.775	0.010	0.194	16

[(1)] Exceeds limits.

Factor Coding: Actual

Response: Day 0 (Log10(CFU/ml))

Design Points:

Above Surface

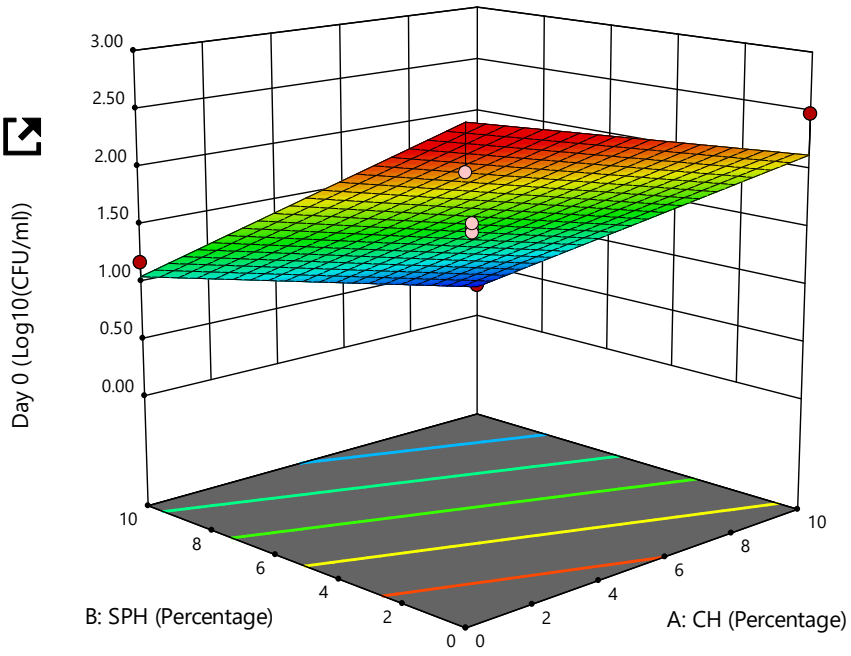
Below Surface

0.30 2.63

Actual Factor:

C = 5

3D Surface



Factor Coding: Actual

Response: Day 0 (Log10(CFU/ml))

Design Points

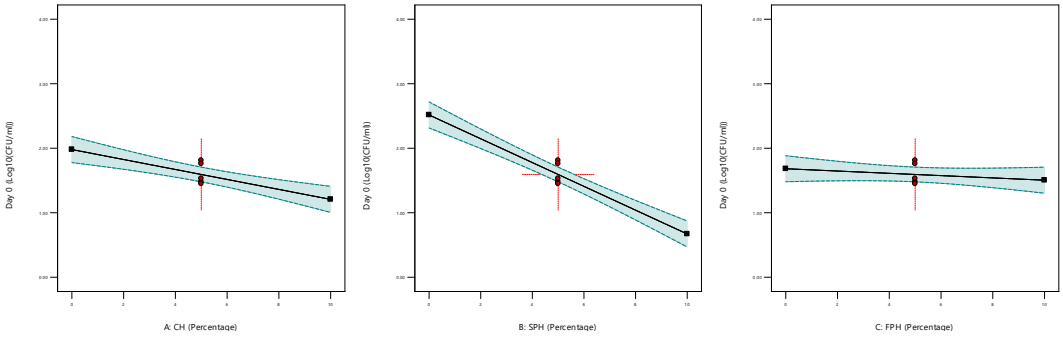
95% CI Bands

Actual Factors:

A = 5

B = 5

C = 5



Fit Summary

Response 2: Day 7

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**0.0004**	**0.8446**	**0.6789**	**0.5822**	**Suggested**
2FI	0.5248	0.8278	0.6628	0.4508	
Quadratic	0.8323	0.6360	0.5713	-0.1564	
Cubic	0.6360		0.4891		**Aliased**

ANOVA for Linear model

Response 2: Day 7

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12.66	3	4.22	12.27	0.0004	significant
A-CH	3.73	1	3.73	10.84	0.0058	
B-SPH	8.88	1	8.88	25.83	0.0002	
C-FPH	0.0528	1	0.0528	0.1536	0.7015	
Residual	4.47	13	0.3439			
Lack of Fit	2.28	9	0.2535	0.4634	0.8446	not significant
Pure Error	2.19	4	0.5471			
Cor Total	17.13	16				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 12.27 implies the model is significant. There is only a 0.04% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.46 implies the Lack of Fit is not significant relative to the pure error. There is a 84.46% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.5864	**R²**	0.7391
Mean	1.52	**Adjusted R²**	0.6789
C.V. %	38.52	**Predicted R²**	0.5822
		Adeq Precision	12.2078

The **Predicted R²** of 0.5822 is in reasonable agreement with the **Adjusted R²** of 0.6789; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 12.208 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.52	1	0.1422	1.22	1.83	
A-CH	-0.6825	1	0.2073	-1.13	-0.2346	1.0000
B-SPH	-1.05	1	0.2073	-1.50	-0.6059	1.0000
C-FPH	-0.0813	1	0.2073	-0.5291	0.3666	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Factor Coding: Actual

Response: Day 7 (Log10(CFU/ml))

Design Points:

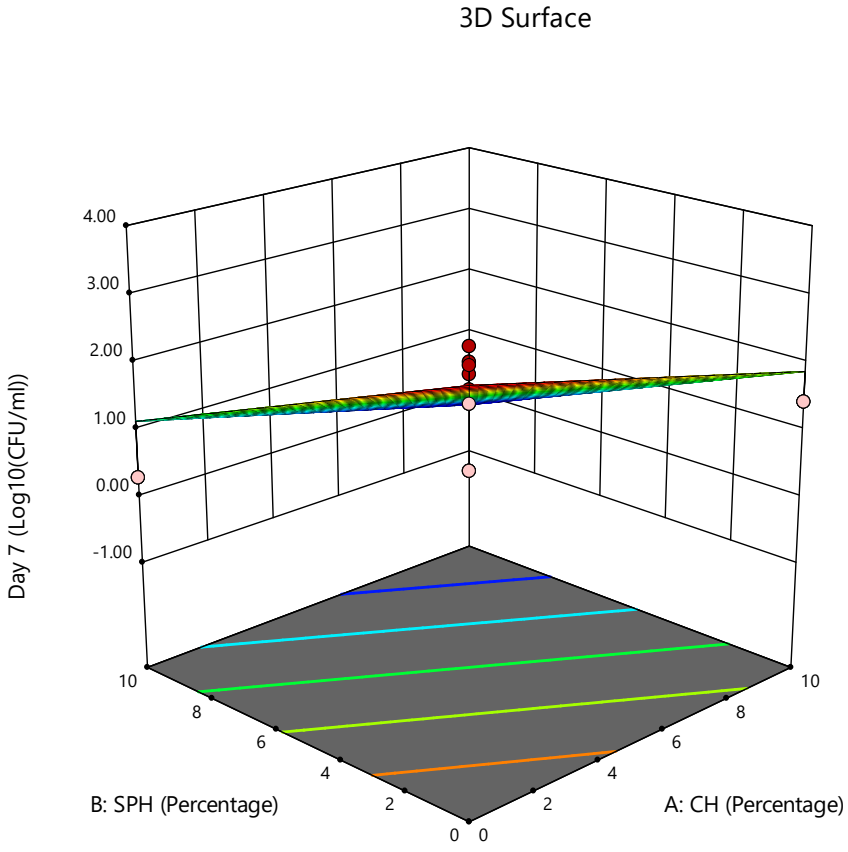
Above Surface

Below Surface

0.30 3.02

Actual Factor:

C = 5



Factor Coding: Actual

Response: Day 7 (Log10(CFU/ml))

Design Points

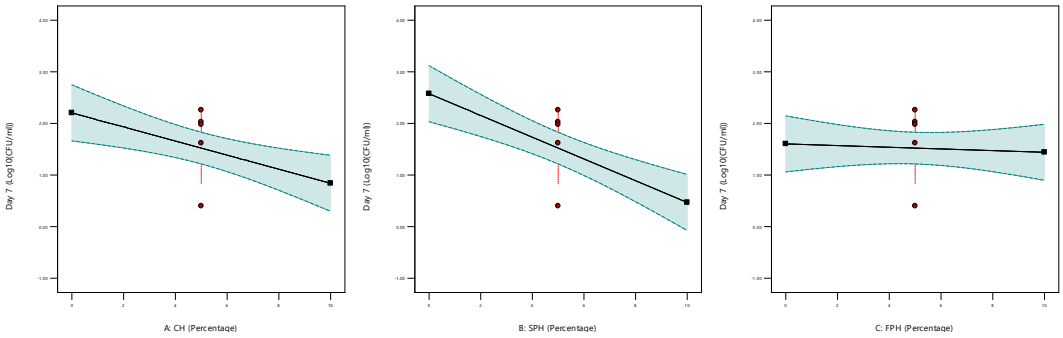
95% CI Bands

Actual Factors:

A = 5

B = 5

C = 5



Fit Summary

Response 3: Day 14

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.8825**	**0.9689**	**0.9607**	**Suggested**
2FI	0.9823	0.7368	0.9602	0.9288	
Quadratic	0.4572	0.7443	0.9599	0.9111	
Cubic	0.7443		0.9469		**Aliased**

Sequential Model Sum of Squares [Type I]

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	36.32	1	36.32			
Linear vs Mean	**6.23**	**3**	**2.08**	**167.15**	**< 0.0001**	**Suggested**
2FI vs Linear	0.0026	3	0.0009	0.0545	0.9823	
Quadratic vs 2FI	0.0468	3	0.0156	0.9741	0.4572	
Cubic vs Quadratic	0.0272	3	0.0091	0.4279	0.7443	Aliased
Residual	0.0849	4	0.0212			
Total	42.72	17	2.51			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.1115**	**0.9747**	**0.9689**	**0.9607**	**0.2514**	**Suggested**
2FI	0.1261	0.9751	0.9602	0.9288	0.4556	
Quadratic	0.1266	0.9825	0.9599	0.9111	0.5687	
Cubic	0.1457	0.9867	0.9469		*	Aliased

- Case(s) with leverage of 1.0000: PRESS statistic not defined.

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.3000	-0.2139	0.5139	0.309	1.054	1.059	0.124	0.708	4
2	0.4800	0.3874	0.0926	0.309	0.190	0.183	0.004	0.122	12
3	0.4000	1.52	-1.12	0.059	-1.973	-2.265	0.061	-0.566	13
4	2.54	2.66	-0.1174	0.309	-0.241	-0.232	0.006	-0.155	9
5	0.4800	0.5499	-0.0699	0.309	-0.143	-0.138	0.002	-0.092	10
6	2.99	2.49	0.4951	0.309	1.016	1.017	0.115	0.680	11
7	1.99	2.12	-0.1336	0.309	-0.274	-0.264	0.008	-0.177	7
8	1.98	1.52	0.4576	0.059	0.804	0.793	0.010	0.198	17
9	0.3000	1.15	-0.8511	0.309	-1.746	-1.917	0.340	-1.281	3
10	1.44	1.89	-0.4536	0.309	-0.930	-0.925	0.097	-0.618	2
11	0.4800	0.7586	-0.2786	0.309	-0.571	-0.556	0.036	-0.372	8
12	3.02	3.26	-0.2386	0.309	-0.489	-0.475	0.027	-0.317	1
13	0.6000	0.9211	-0.3211	0.309	-0.659	-0.644	0.048	-0.430	6
14	2.97	2.29	0.6839	0.309	1.403	1.463	0.220	0.978	5
15	2.03	1.52	0.5076	0.059	0.892	0.885	0.012	0.221	14
16	2.26	1.52	0.7376	0.059	1.297	1.335	0.026	0.334	15
17	1.62	1.52	0.0976	0.059	0.172	0.165	0.000	0.041	16

ANOVA for Linear model

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6.23	3	2.08	167.15	< 0.0001	significant
A-CH	1.28	1	1.28	102.97	< 0.0001	
B-SPH	4.38	1	4.38	352.42	< 0.0001	
C-FPH	0.5724	1	0.5724	46.05	< 0.0001	
Residual	0.1616	13	0.0124			
Lack of Fit	0.0767	9	0.0085	0.4013	0.8825	not significant

Pure Error	0.0849	4	0.0212
Cor Total	6.39	16	

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 167.15 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.40 implies the Lack of Fit is not significant relative to the pure error. There is a 88.25% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1115	**R²**	0.9747
Mean	1.46	**Adjusted R²**	0.9689
C.V. %	7.63	**Predicted R²**	0.9607
		Adeq Precision	42.1584

The **Predicted R²** of 0.9607 is in reasonable agreement with the **Adjusted R²** of 0.9689; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 42.158 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.46	1	0.0270	1.40	1.52	
A-CH	-0.4000	1	0.0394	-0.4852	-0.3148	1.0000
B-SPH	-0.7400	1	0.0394	-0.8252	-0.6548	1.0000
C-FPH	0.2675	1	0.0394	0.1823	0.3527	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.3000	0.3218	-0.0218	0.309	-0.235	-0.226	0.006	-0.151	4
2	1.0000	0.9893	0.0107	0.309	0.116	0.111	0.001	0.074	12
3	1.51	1.46	0.0482	0.059	0.446	0.432	0.003	0.108	13
4	1.99	1.93	0.0557	0.309	0.601	0.586	0.040	0.392	9
5	0.4800	0.4543	0.0257	0.309	0.278	0.268	0.009	0.179	10
6	2.59	2.47	0.1207	0.309	1.303	1.342	0.190	0.897	11
7	2.00	2.13	-0.1293	0.309	-1.395	-1.453	0.217	-0.971	7
8	1.38	1.46	-0.0818	0.059	-0.756	-0.743	0.009	-0.186	17
9	1.20	1.12	0.0782	0.309	0.844	0.834	0.080	0.558	3
10	1.72	1.80	-0.0818	0.309	-0.882	-0.874	0.087	-0.584	2
11	1.26	1.33	-0.0693	0.309	-0.747	-0.734	0.062	-0.491	8
12	2.60	2.60	-0.0018	0.309	-0.019	-0.018	0.000	-0.012	1
13	0.7800	0.7943	-0.0143	0.309	-0.154	-0.148	0.003	-0.099	6
14	1.46	1.59	-0.1343	0.309	-1.449	-1.520	0.234	-1.016	5
15	1.71	1.46	0.2482	0.059	2.295	2.859	0.082	0.715	14
16	1.53	1.46	0.0682	0.059	0.631	0.616	0.006	0.154	15
17	1.34	1.46	-0.1218	0.059	-1.126	-1.138	0.020	-0.285	16

Factor Coding: Actual

Response: Day 14 (Log10(CFU/ml))

Design Points:

Above Surface

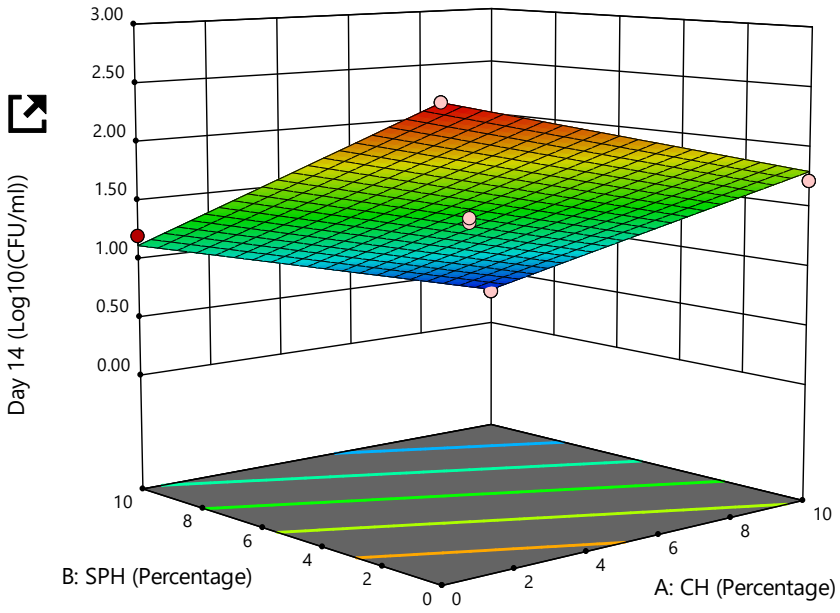
Below Surface

0.30 2.60

Actual Factor:

C = 5

3D Surface



Factor Coding: Actual

Response: Day 14 (Log10(CFU/ml))

Design Points

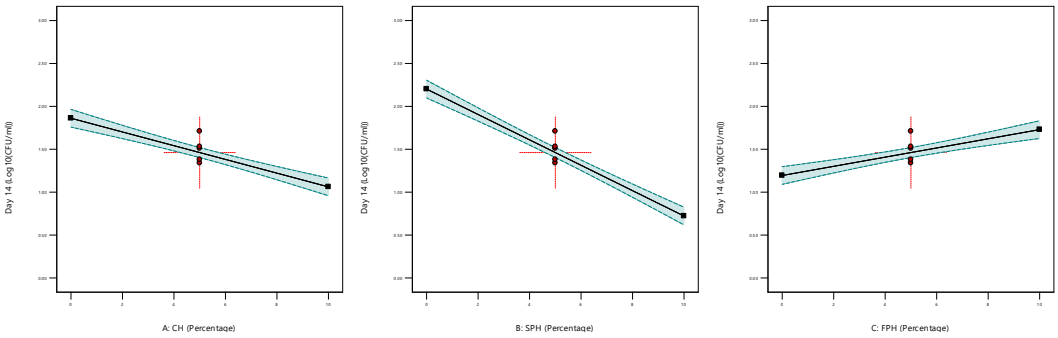
95% CI Bands

Actual Factors:

A = 5

B = 5

C = 5



Fit Summary

Response 4: Day 21

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.2656**	**0.9723**	**0.9590**	**Suggested**
2FI	0.2040	0.3200	0.9768	0.9510	
Quadratic	0.1905	0.4322	0.9825	0.9369	
Cubic	0.4322		0.9835		**Aliased**

Sequential Model Sum of Squares [Type I]

Response 4: Day 21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	36.00	1	36.00			
Linear vs Mean	**8.14**	**3**	**2.71**	**188.06**	**< 0.0001**	**Suggested**
2FI vs Linear	0.0667	3	0.0222	1.84	0.2040	
Quadratic vs 2FI	0.0571	3	0.0190	2.09	0.1905	
Cubic vs Quadratic	0.0295	3	0.0098	1.15	0.4322	Aliased
Residual	0.0343	4	0.0086			
Total	44.33	17	2.61			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

ANOVA for Linear model

Response 4: Day 21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.14	3	2.71	188.06	< 0.0001	significant
A-CH	1.62	1	1.62	112.28	< 0.0001	
B-SPH	6.02	1	6.02	417.25	< 0.0001	
C-FPH	0.5000	1	0.5000	34.65	< 0.0001	
Residual	0.1876	13	0.0144			
Lack of Fit	0.1533	9	0.0170	1.98	0.2656	not significant

Pure Error	0.0343	4	0.0086
Cor Total	8.33	16	

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 188.06 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.98 implies the Lack of Fit is not significant relative to the pure error. There is a 26.56% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1201	**R²**	0.9775
Mean	1.46	**Adjusted R²**	0.9723
C.V. %	8.25	**Predicted R²**	0.9590
		Adeq Precision	45.2232

The **Predicted R²** of 0.9590 is in reasonable agreement with the **Adjusted R²** of 0.9723; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 45.223 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Coded Factors

Day 21 =
+1.46
-0.4500 A
-0.8675 B
+0.2500 C

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.3000	0.1378	0.1622	0.309	1.624	1.748	0.295	1.168	4
2	0.7800	0.8378	-0.0578	0.309	-0.579	-0.563	0.037	-0.377	12
3	1.36	1.46	-0.0953	0.059	-0.818	-0.807	0.010	-0.202	13
4	1.93	2.07	-0.1428	0.309	-1.430	-1.497	0.228	-1.000	9
5	0.3000	0.3378	-0.0378	0.309	-0.378	-0.366	0.016	-0.244	10
6	2.64	2.57	0.0672	0.309	0.673	0.658	0.051	0.440	11
7	2.08	2.16	-0.0753	0.309	-0.754	-0.741	0.063	-0.495	7
8	1.52	1.46	0.0647	0.059	0.555	0.540	0.005	0.135	17
9	1.04	1.04	0.0022	0.309	0.022	0.021	0.000	0.014	3
10	1.94	1.87	0.0672	0.309	0.673	0.658	0.051	0.440	2
11	1.32	1.26	0.0647	0.309	0.648	0.633	0.047	0.423	8
12	2.85	2.77	0.0772	0.309	0.773	0.760	0.067	0.508	1
13	0.7000	0.7553	-0.0553	0.309	-0.554	-0.538	0.034	-0.360	6
14	1.89	1.66	0.2347	0.309	2.350	2.978	0.617	1.990[1]	5
15	1.28	1.46	-0.1753	0.059	-1.504	-1.590	0.035	-0.398	14
16	1.36	1.46	-0.0953	0.059	-0.818	-0.807	0.010	-0.202	15
17	1.45	1.46	-0.0053	0.059	-0.045	-0.044	0.000	-0.011	16

[1] Exceeds limits.

Factor Coding: Actual

Response: Day 21 (Log10(CFU/ml))

Design Points:

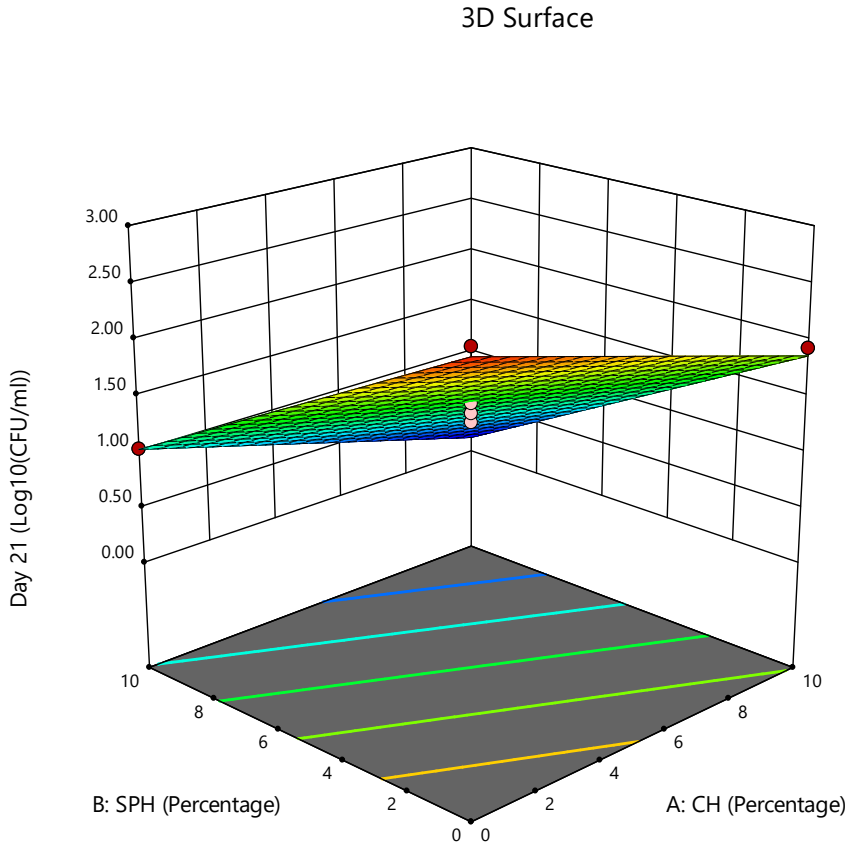
Above Surface

Below Surface

0.30 2.85

Actual Factor:

C = 5



Factor Coding: Actual

Response: Day 21 (Log10(CFU/ml))

Design Points

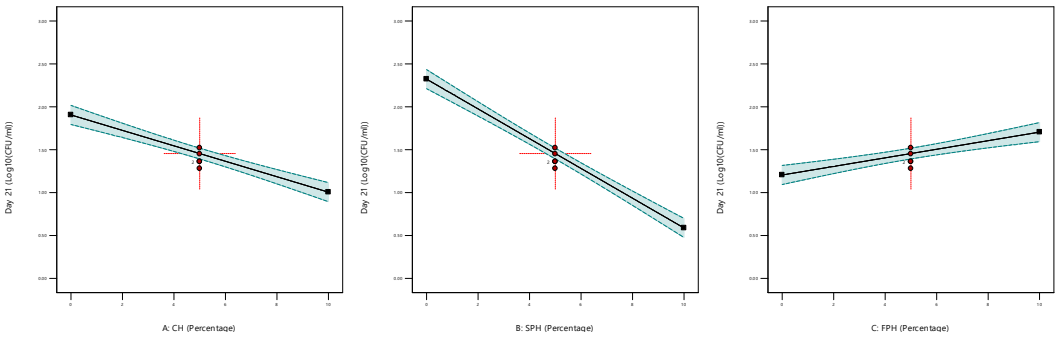
95% CI Bands

Actual Factors:

A = 5

B = 5

C = 5



Fit Summary

Response 5: Day 28

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.8646**	**0.9036**	**0.8865**	**Suggested**
2FI	0.9984	0.7013	0.8750	0.8086	
Quadratic	0.5076	0.6568	0.8693	0.6593	
Cubic	0.6568		0.8409		**Aliased**

Sequential Model Sum of Squares [Type I]

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	28.99	1	28.99			
Linear vs Mean	**12.66**	**3**	**4.22**	**50.97**	**< 0.0001**	**Suggested**
2FI vs Linear	0.0034	3	0.0011	0.0107	0.9984	
Quadratic vs 2FI	0.2874	3	0.0958	0.8536	0.5076	
Cubic vs Quadratic	0.2391	3	0.0797	0.5835	0.6568	Aliased
Residual	0.5464	4	0.1366			
Total	42.73	17	2.51			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

ANOVA for Linear model

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12.66	3	4.22	50.97	< 0.0001	significant
A-CH	2.17	1	2.17	26.25	0.0002	
B-SPH	9.72	1	9.72	117.45	< 0.0001	
C-FPH	0.7626	1	0.7626	9.21	0.0096	
Residual	1.08	13	0.0828			
Lack of Fit	0.5299	9	0.0589	0.4311	0.8646	not significant

Pure Error	0.5464	4	0.1366
Cor Total	13.74	16	

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 50.97 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.43 implies the Lack of Fit is not significant relative to the pure error. There is a 86.46% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.2877	**R²**	0.9216
Mean	1.31	**Adjusted R²**	0.9036
C.V. %	22.03	**Predicted R²**	0.8865
		Adeq Precision	23.2670

The **Predicted R²** of 0.8865 is in reasonable agreement with the **Adjusted R²** of 0.9036; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 23.267 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.0000	-0.3179	0.3179	0.309	1.329	1.373	0.197	0.918	4
2	0.3000	0.5121	-0.2121	0.309	-0.887	-0.879	0.088	-0.588	12
3	0.4800	1.31	-0.8259	0.059	-2.959	-4.973[(1)]	0.137	-1.243	13
4	2.32	2.10	0.2204	0.309	0.921	0.915	0.095	0.612	9
5	0.0000	-0.1054	0.1054	0.309	0.440	0.426	0.022	0.285	10
6	2.73	2.72	0.0129	0.309	0.054	0.052	0.000	0.035	11
7	2.37	2.14	0.2341	0.309	0.979	0.977	0.107	0.653	7
8	1.40	1.31	0.0941	0.059	0.337	0.325	0.002	0.081	17
9	0.9000	0.7246	0.1754	0.309	0.733	0.719	0.060	0.481	3
10	2.04	1.89	0.1529	0.309	0.639	0.624	0.046	0.417	2
11	1.20	1.09	0.1066	0.309	0.446	0.432	0.022	0.288	8
12	2.93	2.93	0.0004	0.309	0.002	0.001	0.000	0.001	1
13	0.3000	0.4759	-0.1759	0.309	-0.735	-0.722	0.060	-0.482	6
14	1.51	1.52	-0.0084	0.309	-0.035	-0.034	0.000	-0.023	5
15	1.18	1.31	-0.1259	0.059	-0.451	-0.437	0.003	-0.109	14
16	1.20	1.31	-0.1059	0.059	-0.379	-0.366	0.002	-0.092	15
17	1.34	1.31	0.0341	0.059	0.122	0.117	0.000	0.029	16

[(1)] Observation with |External Stud. Residuals| > 3.72

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.31	1	0.0698	1.16	1.46	
A-CH	-0.5212	1	0.1017	-0.7410	-0.3015	1.0000
B-SPH	-1.10	1	0.1017	-1.32	-0.8827	1.0000
C-FPH	0.3088	1	0.1017	0.0890	0.5285	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points:

Above Surface

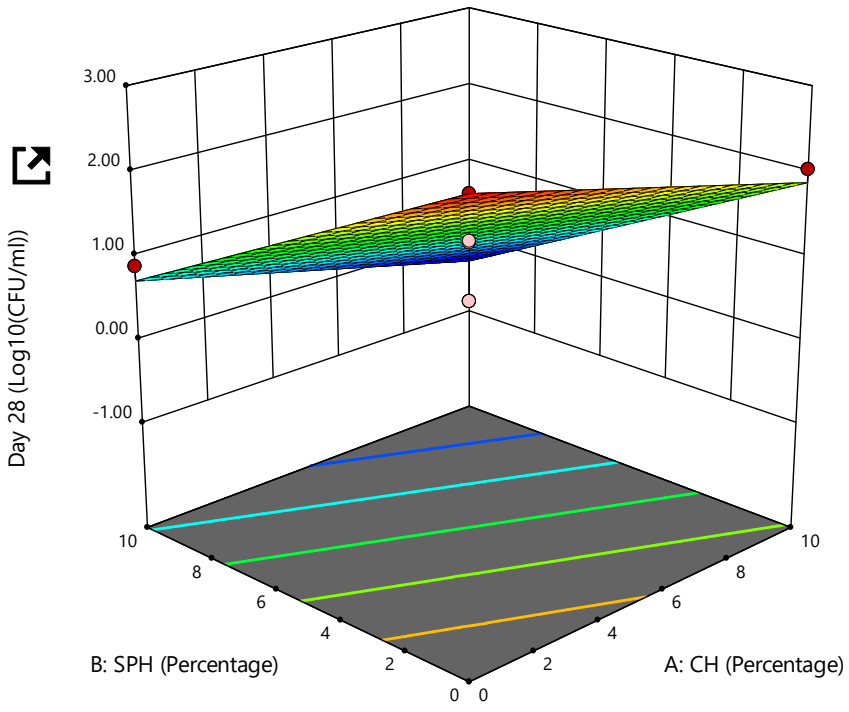
Below Surface

0.00 2.93

Actual Factor:

C = 5

3D Surface



Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points

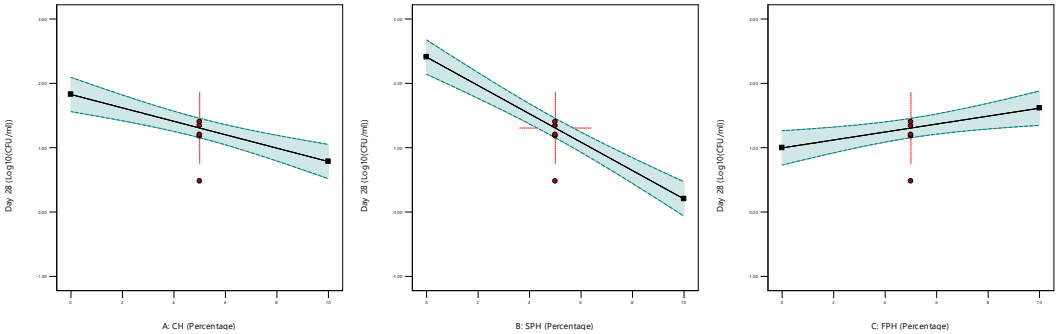
95% CI Bands

Actual Factors:

A = 5

B = 5

C = 5



Appendix 4*:* Raw data from RSM software for protectants study of *S. enterica*

Fit Summary

Response 1: Day 0

Source	Sequential p-value	Lack of Fit p-value	Adjusted R²	Predicted R²	
Linear	**< 0.0001**	**0.9836**	**0.9162**	**0.9035**	**Suggested**
2FI	0.8737	0.9638	0.9012	0.8623	
Quadratic	0.5019	0.9685	0.8985	0.8402	
Cubic	0.9099	0.8451	0.8396	0.5511	**Aliased**

Model Summary Statistics

Source	Std. Dev.	R²	Adjusted R²	Predicted R²	PRESS	
Linear	**0.0874**	**0.9282**	**0.9162**	**0.9035**	**0.2460**	**Suggested**
2FI	0.0949	0.9365	0.9012	0.8623	0.3512	
Quadratic	0.0962	0.9492	0.8985	0.8402	0.4075	
Cubic	0.1209	0.9656	0.8396	0.5511	1.14	Aliased

Focus on the model maximizing the **Adjusted R²** and the **Predicted R²**.

ANOVA for Linear model

Response 1: Day 0

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.37	4	0.5918	77.54	< 0.0001	significant
A-Glucose	1.75	1	1.75	229.05	< 0.0001	
B-Glycerol	0.3434	1	0.3434	45.00	< 0.0001	
C-Trehalose	0.0432	1	0.0432	5.66	0.0257	
D-BSA	0.2324	1	0.2324	30.45	< 0.0001	
Residual	0.1832	24	0.0076			
Lack of Fit	0.1026	20	0.0051	0.2545	0.9836	not significant
Pure Error	0.0806	4	0.0202			
Cor Total	2.55	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 77.54 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.25 implies the Lack of Fit is not significant relative to the pure error. There is a 98.36% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.0874	**R^2**	0.9282
Mean	1.44	**Adjusted R^2**	0.9162
C.V. %	6.06	**Predicted R^2**	0.9035
		Adeq Precision	30.3704

The **Predicted R^2** of 0.9035 is in reasonable agreement with the **Adjusted R^2** of 0.9162; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 30.370 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.44	1	0.0162	1.41	1.48	
A-Glucose	-0.3817	1	0.0252	-0.4337	-0.3296	1.0000
B-Glycerol	-0.1692	1	0.0252	-0.2212	-0.1171	1.0000
C-Trehalose	-0.0600	1	0.0252	-0.1120	-0.0080	1.0000
D-BSA	-0.1392	1	0.0252	-0.1912	-0.0871	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

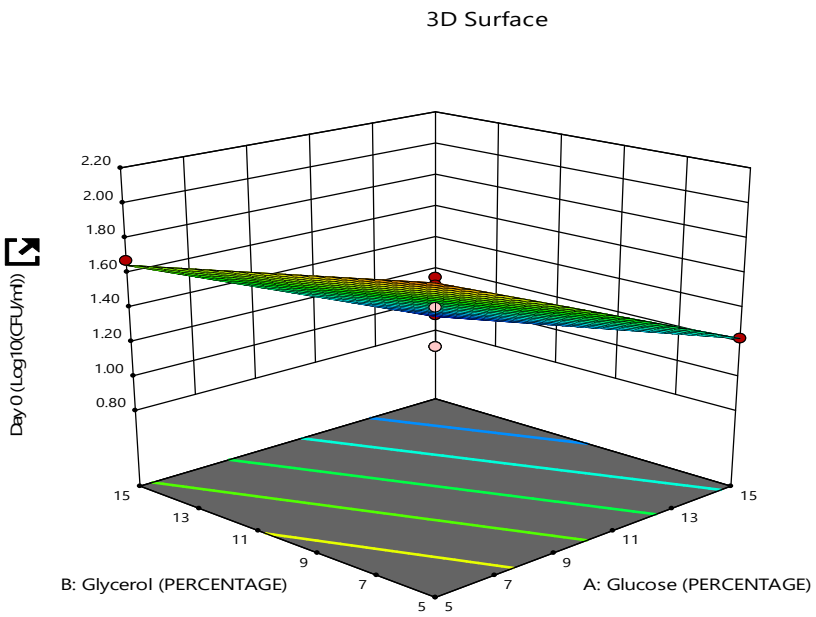
Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.15	1.96	0.1874	0.201	2.401	2.696	0.290	1.353(1)	9
2	1.08	1.00	0.0799	0.201	1.024	1.025	0.053	0.514	20
3	1.54	1.44	0.0983	0.034	1.145	1.153	0.009	0.218	25
4	1.30	1.20	0.1008	0.201	1.291	1.310	0.084	0.657	10
5	1.54	1.64	-0.1009	0.201	-1.292	-1.311	0.084	-0.658	5
6	1.92	1.88	0.0366	0.201	0.469	0.461	0.011	0.231	17
7	1.20	1.21	-0.0126	0.201	-0.161	-0.158	0.001	-0.079	16
8	1.41	1.44	-0.0317	0.034	-0.370	-0.363	0.001	-0.069	26
9	1.34	1.41	-0.0717	0.201	-0.919	-0.915	0.042	-0.459	22
10	1.36	1.36	-0.0026	0.201	-0.033	-0.032	0.000	-0.016	7
11	1.08	1.12	-0.0401	0.201	-0.513	-0.505	0.013	-0.253	18
12	1.18	1.44	-0.2617	0.034	-3.049	-3.813(2)	0.066	-0.721	29
13	1.45	1.55	-0.1009	0.201	-1.292	-1.311	0.084	-0.658	15
14	2.02	1.99	0.0274	0.201	0.351	0.345	0.006	0.173	1
15	1.68	1.65	0.0258	0.201	0.330	0.324	0.005	0.163	3
16	1.72	1.75	-0.0301	0.201	-0.385	-0.378	0.007	-0.190	21
17	1.11	1.13	-0.0234	0.201	-0.300	-0.294	0.005	-0.147	24
18	1.48	1.47	0.0083	0.201	0.106	0.104	0.001	0.052	23
19	1.78	1.76	0.0166	0.201	0.213	0.208	0.002	0.105	19
20	1.45	1.52	-0.0709	0.201	-0.908	-0.904	0.042	-0.454	6
21	1.62	1.68	-0.0642	0.201	-0.823	-0.817	0.034	-0.410	11
22	1.26	1.24	0.0174	0.201	0.223	0.219	0.003	0.110	8
23	1.70	1.67	0.0291	0.201	0.373	0.366	0.007	0.184	13
24	1.0000	0.9209	0.0791	0.201	1.013	1.014	0.052	0.509	12
25	1.23	1.23	0.0008	0.201	0.010	0.010	0.000	0.005	2
26	1.34	1.33	0.0074	0.201	0.095	0.093	0.000	0.047	14
27	1.48	1.44	0.0383	0.034	0.446	0.438	0.001	0.083	27
28	0.9000	0.8909	0.0091	0.201	0.117	0.114	0.001	0.057	4
29	1.49	1.44	0.0483	0.034	0.562	0.554	0.002	0.105	28

(1) Exceeds limits.

(2) Observation with |External Stud. Residuals| > 3.55

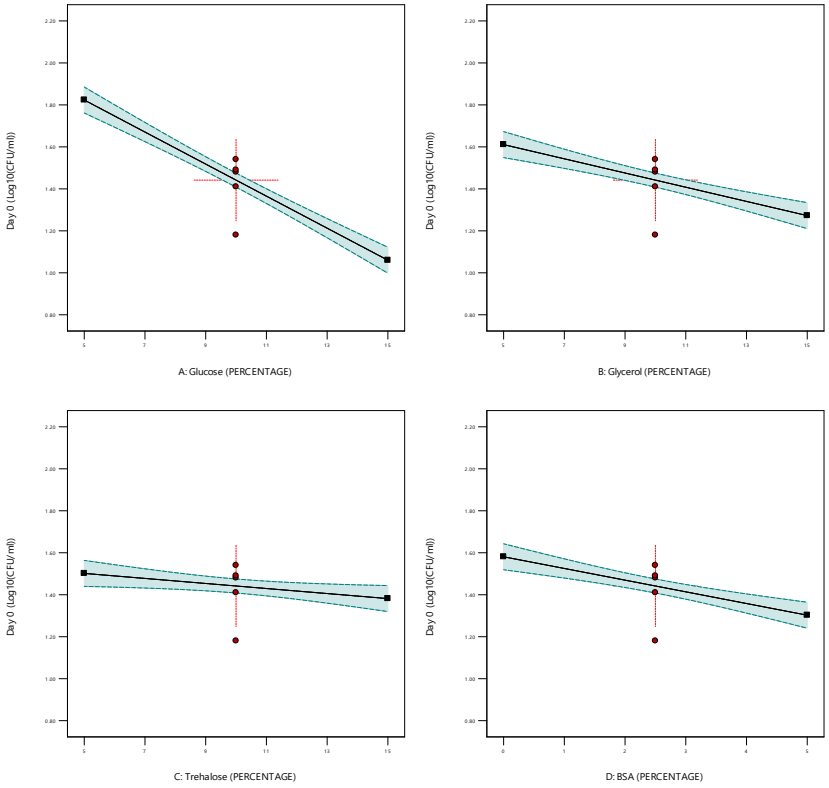
Factor Coding: Actual
Response: Day 0 (Log10(CFU/ml))
Design Points:
Above Surface
Below Surface
0.90 2.15

Actual Factors:
C = 10
D = 2.5



Factor Coding: Actual
Response: Day 0 (Log10(CFU/ml))
Design Points
95% CI Bands

Actual Factors:
A = 10
B = 10
C = 10
D = 2.5



Fit Summary

Response 2: Day 7

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.9601**	**0.9007**	**0.8826**	**Suggested**
2FI	0.8591	0.9244	0.8837	0.8246	
Quadratic	0.7480	0.8842	0.8686	0.7576	
Cubic	0.8224	0.6734	0.8157	-0.0711	**Aliased**

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.1238**	**0.9149**	**0.9007**	**0.8826**	**0.5074**	**Suggested**
2FI	0.1340	0.9252	0.8837	0.8246	0.7581	
Quadratic	0.1424	0.9343	0.8686	0.7576	1.05	
Cubic	0.1687	0.9605	0.8157	-0.0711	4.63	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	**0.2279**	**20**	**0.0114**	**0.3253**	**0.9601**	**Suggested**
2FI	0.1831	14	0.0131	0.3734	0.9244	
Quadratic	0.1439	10	0.0144	0.4108	0.8842	
Cubic	0.0306	2	0.0153	0.4372	0.6734	Aliased
Pure Error	0.1401	4	0.0350			

The selected model should have insignificant lack-of-fit.

ANOVA for Linear model

Response 2: Day 7

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.95	4	0.9887	64.48	< 0.0001	significant

A-Glucose	2.85	1	2.85	185.99	< 0.0001	
B-Glycerol	0.5590	1	0.5590	36.46	< 0.0001	
C-Trehalose	0.1801	1	0.1801	11.74	0.0022	
D-BSA	0.3640	1	0.3640	23.74	< 0.0001	
Residual	0.3680	24	0.0153			
Lack of Fit	0.2279	20	0.0114	0.3253	0.9601	not significant
Pure Error	0.1401	4	0.0350			
Cor Total	4.32	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 64.48 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.33 implies the Lack of Fit is not significant relative to the pure error. There is a 96.01% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1238	**R²**	0.9149
Mean	1.33	**Adjusted R²**	0.9007
C.V. %	9.33	**Predicted R²**	0.8826
		Adeq Precision	27.3577

The **Predicted R²** of 0.8826 is in reasonable agreement with the **Adjusted R²** of 0.9007; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 27.358 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.33	1	0.0230	1.28	1.37	
A-Glucose	-0.4875	1	0.0357	-0.5613	-0.4137	1.0000
B-Glycerol	-0.2158	1	0.0357	-0.2896	-0.1421	1.0000
C-Trehalose	-0.1225	1	0.0357	-0.1963	-0.0487	1.0000
D-BSA	-0.1742	1	0.0357	-0.2479	-0.1004	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.12	1.99	0.1311	0.201	1.184	1.195	0.071	0.600	9
2	0.7800	0.7172	0.0628	0.201	0.567	0.559	0.016	0.280	20
3	1.45	1.33	0.1228	0.034	1.009	1.009	0.007	0.191	25
4	1.18	1.01	0.1661	0.201	1.501	1.543	0.113	0.774	10
5	1.64	1.62	0.0161	0.201	0.145	0.142	0.001	0.071	5
6	1.99	1.94	0.0528	0.201	0.477	0.469	0.011	0.235	17
7	1.0000	0.9889	0.0111	0.201	0.100	0.098	0.001	0.049	16
8	1.34	1.33	0.0128	0.034	0.105	0.103	0.000	0.019	26
9	1.20	1.29	-0.0856	0.201	-0.773	-0.767	0.030	-0.385	22
10	1.34	1.28	0.0644	0.201	0.582	0.574	0.017	0.288	7
11	0.7000	0.9622	-0.2622	0.201	-2.369	-2.650	0.283	-1.330[1]	18
12	1.0000	1.33	-0.3272	0.034	-2.689	-3.150	0.052	-0.595	29
13	1.34	1.42	-0.0806	0.201	-0.728	-0.721	0.027	-0.362	15
14	2.04	2.03	0.0094	0.201	0.085	0.083	0.000	0.042	1
15	1.63	1.60	0.0311	0.201	0.281	0.275	0.004	0.138	3
16	1.62	1.72	-0.0972	0.201	-0.879	-0.874	0.039	-0.439	21
17	0.8500	0.9372	-0.0872	0.201	-0.788	-0.782	0.031	-0.392	24
18	1.38	1.37	0.0111	0.201	0.100	0.098	0.001	0.049	23
19	1.75	1.69	0.0578	0.201	0.522	0.514	0.014	0.258	19
20	1.28	1.38	-0.0989	0.201	-0.894	-0.890	0.040	-0.446	6

21	1.58	1.64	-0.0606	0.201	-0.547	-0.539	0.015	-0.271	11
22	0.9500	1.03	-0.0806	0.201	-0.728	-0.721	0.027	-0.362	8
23	1.62	1.67	-0.0456	0.201	-0.412	-0.405	0.009	-0.203	13
24	0.8500	0.6656	0.1844	0.201	1.666	1.735	0.140	0.870	12
25	1.15	1.06	0.0944	0.201	0.853	0.848	0.037	0.426	2
26	1.28	1.23	0.0461	0.201	0.416	0.409	0.009	0.205	14
27	1.38	1.33	0.0528	0.034	0.434	0.426	0.001	0.081	27
28	0.6000	0.6239	-0.0239	0.201	-0.216	-0.212	0.002	-0.106	4
29	1.45	1.33	0.1228	0.034	1.009	1.009	0.007	0.191	28

(1) Exceeds limits.

Factor Coding: Actual

Response: Day 7 (Log10 (cFU/ml))

Design Points:

Above Surface

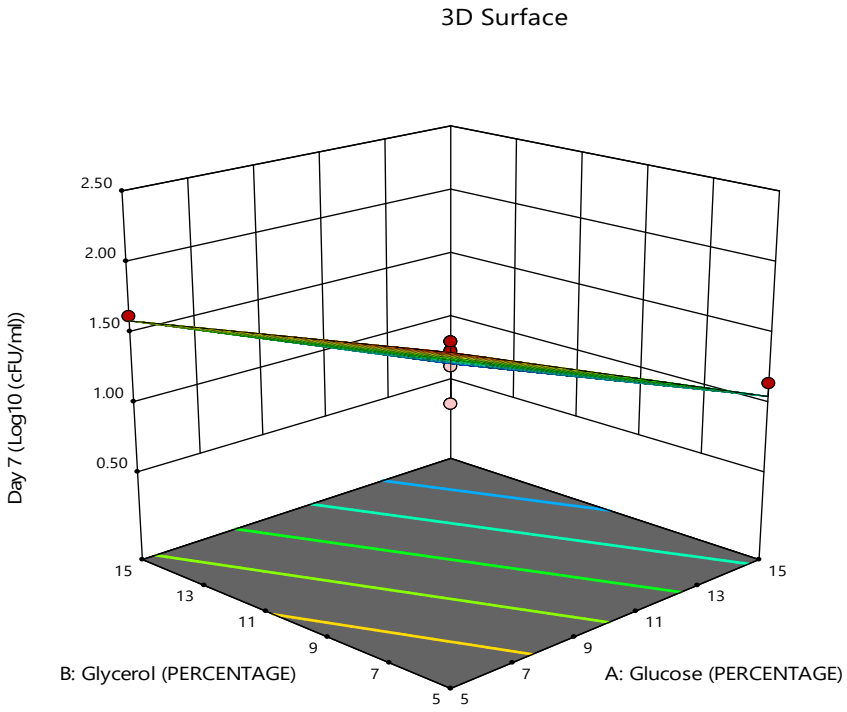
Below Surface

0.60 2.12

Actual Factors:

C = 10

D = 2.5



Factor Coding: Actual

Response: Day 7 (Log10 (cFU/ml))

Design Points

95% CI Bands

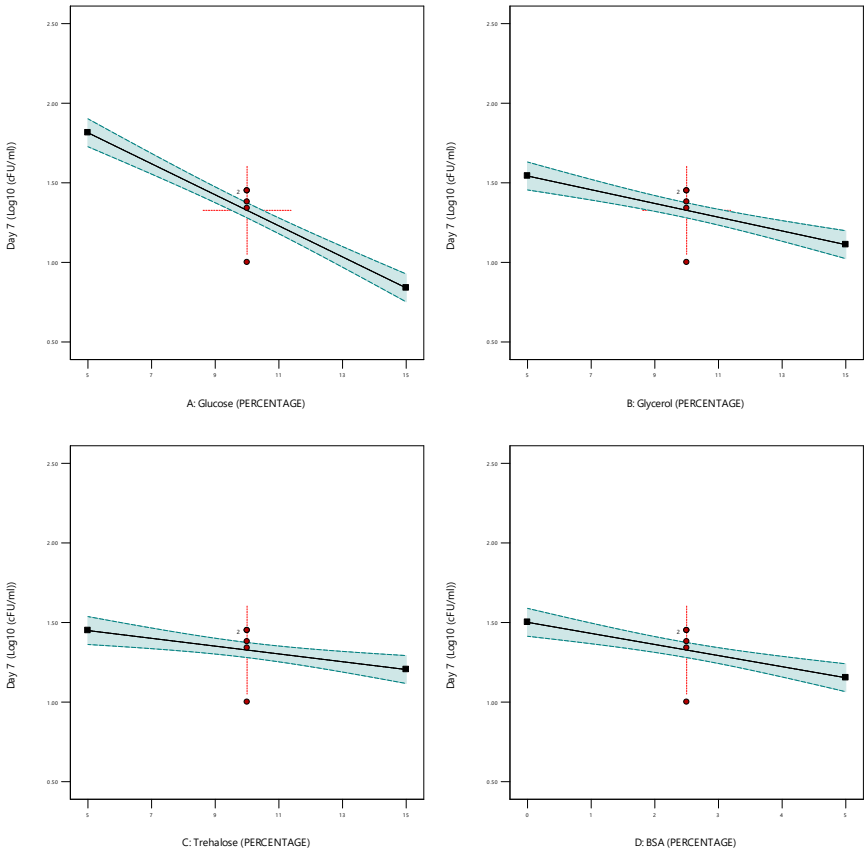
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 3: Day 14

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.1578**	**0.8944**	**0.8629**	**Suggested**
2FI	0.7105	0.1260	0.8834	0.7729	
Quadratic	**0.0248**	**0.2621**	**0.9291**	**0.8205**	**Suggested**
Cubic	0.2140	0.3605	0.9542	0.4261	**Aliased**

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	**0.6336**	**20**	**0.0317**	**2.87**	**0.1578**	**Suggested**
2FI	0.5172	14	0.0369	3.35	0.1260	
Quadratic	**0.2214**	**10**	**0.0221**	**2.01**	**0.2621**	**Suggested**
Cubic	0.0294	2	0.0147	1.33	0.3605	Aliased
Pure Error	0.0441	4	0.0110			

The selected model should have insignificant lack-of-fit.

ANOVA for Quadratic model

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.22	14	0.5158	27.20	< 0.0001	significant
A-Glucose	4.06	1	4.06	214.11	< 0.0001	
B-Glycerol	1.10	1	1.10	57.91	< 0.0001	
C-Trehalose	0.7701	1	0.7701	40.61	< 0.0001	
D-BSA	0.8802	1	0.8802	46.42	< 0.0001	
AB	0.0196	1	0.0196	1.03	0.3266	
AC	0.0272	1	0.0272	1.44	0.2507	
AD	0.0020	1	0.0020	0.1068	0.7487	
BC	0.0306	1	0.0306	1.62	0.2245	
BD	0.0144	1	0.0144	0.7594	0.3982	
CD	0.0225	1	0.0225	1.19	0.2944	
A^2	0.0751	1	0.0751	3.96	0.0665	
B^2	0.0284	1	0.0284	1.50	0.2412	
C^2	0.0748	1	0.0748	3.95	0.0669	
D^2	0.0875	1	0.0875	4.62	0.0497	
Residual	0.2655	14	0.0190			
Lack of Fit	0.2214	10	0.0221	2.01	0.2621	not significant
Pure Error	0.0441	4	0.0110			
Cor Total	7.49	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 27.20 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 2.01 implies the Lack of Fit is not significant relative to the pure error. There is a 26.21% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1377	**R^2**	0.9645
Mean	1.11	**Adjusted R^2**	0.9291
C.V. %	12.42	**Predicted R^2**	0.8205
		Adeq Precision	18.0110

The **Predicted R^2** of 0.8205 is in reasonable agreement with the **Adjusted R^2** of 0.9291; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 18.011 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.18	1	0.0616	1.05	1.32	
A-Glucose	-0.5817	1	0.0398	-0.6669	-0.4964	1.0000
B-Glycerol	-0.3025	1	0.0398	-0.3878	-0.2172	1.0000
C-Trehalose	-0.2533	1	0.0398	-0.3386	-0.1681	1.0000
D-BSA	-0.2708	1	0.0398	-0.3561	-0.1856	1.0000
AB	-0.0700	1	0.0689	-0.2177	0.0777	1.0000
AC	-0.0825	1	0.0689	-0.2302	0.0652	1.0000
AD	0.0225	1	0.0689	-0.1252	0.1702	1.0000
BC	-0.0875	1	0.0689	-0.2352	0.0602	1.0000

BD	-0.0600	1	0.0689	-0.2077	0.0877	1.0000
CD	-0.0750	1	0.0689	-0.2227	0.0727	1.0000
A^2	0.1076	1	0.0541	-0.0084	0.2235	1.08
B^2	-0.0662	1	0.0541	-0.1821	0.0498	1.08
C^2	-0.1074	1	0.0541	-0.2234	0.0085	1.08
D^2	-0.1162	1	0.0541	-0.2321	-0.0002	1.08

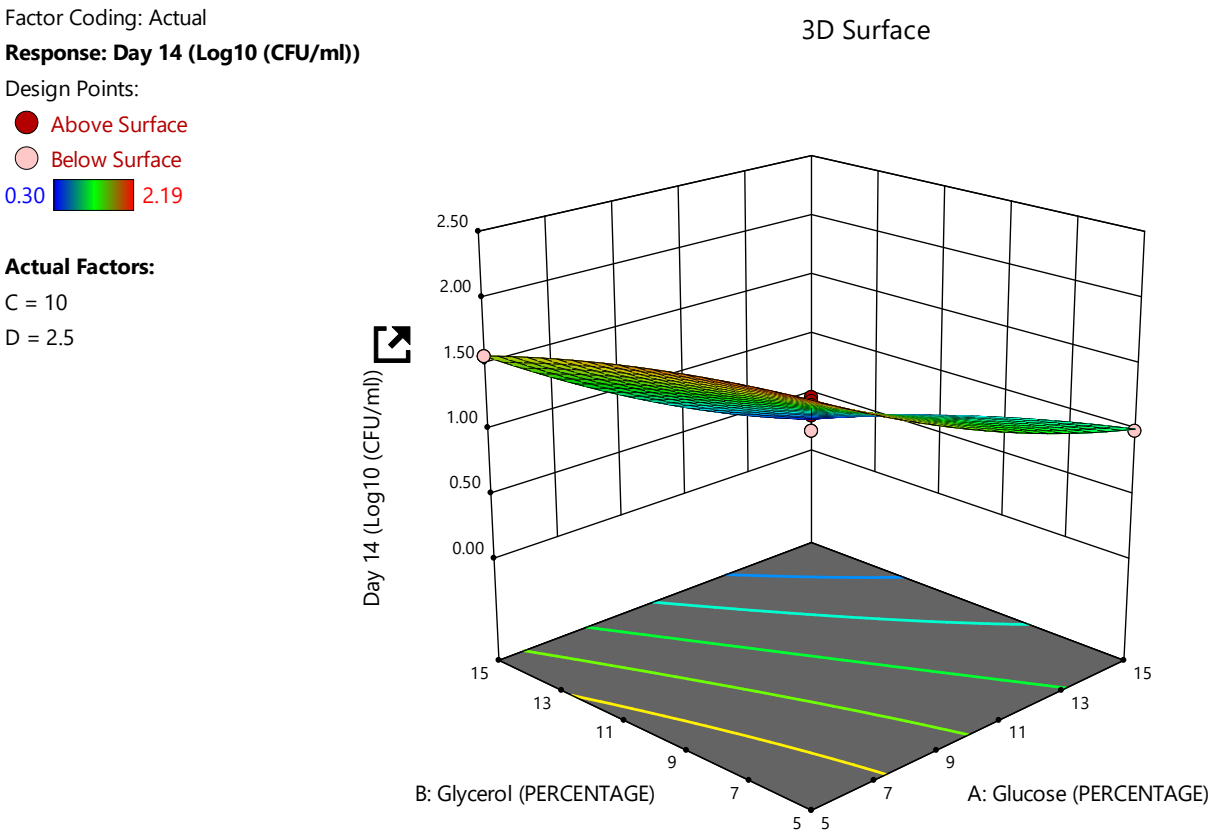
The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.19	2.05	0.1396	0.583	1.570	1.667	0.230	1.973	9
2	0.3000	0.2667	0.0333	0.583	0.375	0.363	0.013	0.430	20
3	1.23	1.18	0.0460	0.200	0.373	0.362	0.002	0.181	25
4	1.0000	0.8421	0.1579	0.583	1.777	1.945	0.295	2.302(1)	10
5	1.45	1.41	0.0404	0.583	0.455	0.441	0.019	0.522	5
6	1.83	1.94	-0.1067	0.583	-1.200	-1.221	0.134	-1.445	17
7	0.4800	0.3671	0.1129	0.583	1.270	1.301	0.151	1.540	16
8	1.26	1.18	0.0760	0.200	0.617	0.603	0.006	0.301	26
9	0.9000	1.03	-0.1300	0.583	-1.463	-1.531	0.200	-1.812	22
10	1.20	1.02	0.1821	0.583	2.048	2.359	0.392	2.791(1)	7
11	0.7800	0.9383	-0.1583	0.583	-1.781	-1.952	0.296	-2.309(1)	18
12	1.0000	1.18	-0.1840	0.200	-1.494	-1.570	0.037	-0.785	29
13	1.18	1.15	0.0329	0.583	0.370	0.359	0.013	0.424	15
14	1.99	2.04	-0.0496	0.583	-0.558	-0.544	0.029	-0.643	1
15	1.57	1.57	-0.0046	0.583	-0.052	-0.050	0.000	-0.059	3
16	1.51	1.52	-0.0050	0.583	-0.056	-0.054	0.000	-0.064	21
17	0.3000	0.3683	-0.0683	0.583	-0.769	-0.757	0.055	-0.896	24
18	1.15	1.09	0.0567	0.583	0.638	0.623	0.038	0.738	23
19	1.68	1.60	0.0850	0.583	0.956	0.953	0.085	1.128	19
20	0.8500	1.05	-0.2029	0.583	-2.283	-2.776	0.486	-3.285(1)	6
21	1.40	1.46	-0.0638	0.583	-0.717	-0.704	0.048	-0.833	11
22	0.3000	0.3613	-0.0613	0.583	-0.689	-0.676	0.044	-0.799	8

23	1.46	1.48	-0.0188	0.583	-0.211	-0.204	0.004	-0.241	13
24	0.3000	0.3454	-0.0454	0.583	-0.511	-0.497	0.024	-0.588	12
25	1.0000	1.02	-0.0162	0.583	-0.183	-0.176	0.003	-0.209	2
26	1.11	1.05	0.0612	0.583	0.689	0.676	0.044	0.799	14
27	1.23	1.18	0.0460	0.200	0.373	0.362	0.002	0.181	27
28	0.3000	0.2713	0.0287	0.583	0.323	0.313	0.010	0.370	4
29	1.20	1.18	0.0160	0.200	0.130	0.125	0.000	0.063	28

(1) Exceeds limits.



Factor Coding: Actual

Response: Day 14 (Log10 (CFU/ml))

Design Points

95% CI Bands

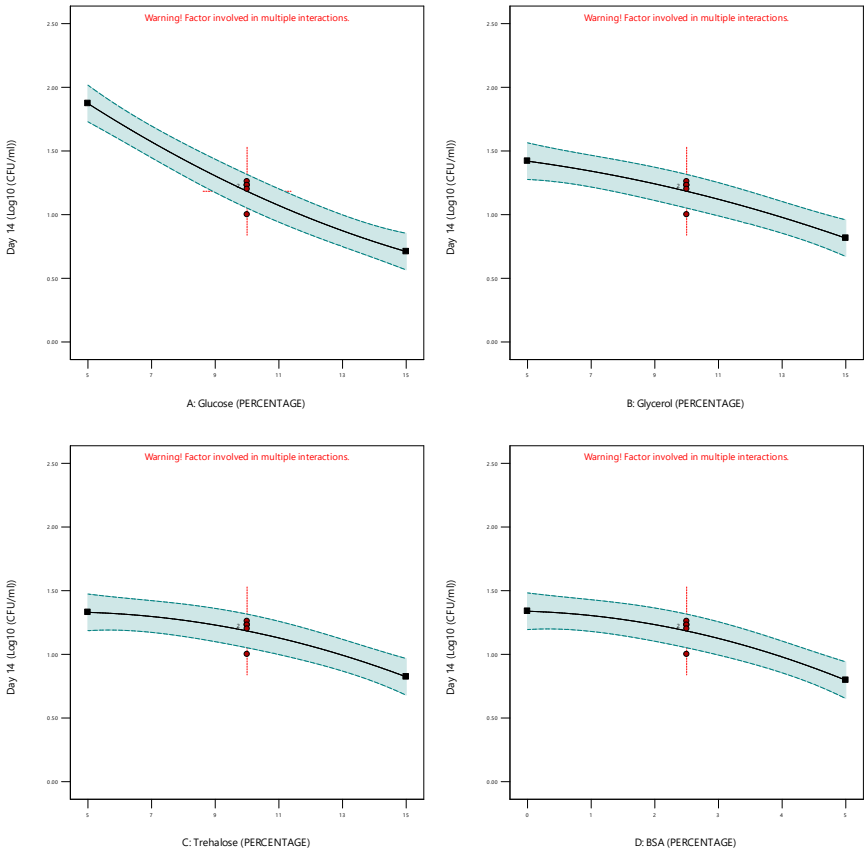
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 4: Day21

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.1410**	**0.8422**	**0.7951**	**Suggested**
2FI	0.8615	0.1011	0.8150	0.6374	
Quadratic	0.1027	0.1467	0.8581	0.6259	
Cubic	0.1181	0.2904	0.9289	-0.0252	**Aliased**

Sequential Model Sum of Squares [Type I]

Response 4: Day21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	31.06	1	31.06			
Linear vs Mean	**7.25**	**4**	**1.81**	**38.36**	**< 0.0001**	**Suggested**
2FI vs Linear	0.1370	6	0.0228	0.4119	0.8615	
Quadratic vs 2FI	0.4026	4	0.1007	2.37	0.1027	
Cubic vs Quadratic	0.4671	8	0.0584	2.74	0.1181	Aliased
Residual	0.1279	6	0.0213			
Total	39.44	29	1.36			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.2174**	**0.8647**	**0.8422**	**0.7951**	**1.72**	**Suggested**
2FI	0.2354	0.8811	0.8150	0.6374	3.04	
Quadratic	0.2062	0.9291	0.8581	0.6259	3.14	
Cubic	0.1460	0.9848	0.9289	-0.0252	8.60	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

ANOVA for Linear model

Response 4: Day21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.25	4	1.81	38.36	< 0.0001	significant
A-Glucose	4.49	1	4.49	94.97	< 0.0001	
B-Glycerol	1.13	1	1.13	24.00	< 0.0001	
C-Trehalose	0.6721	1	0.6721	14.22	0.0009	
D-BSA	0.9577	1	0.9577	20.26	0.0001	
Residual	1.13	24	0.0473			
Lack of Fit	1.07	20	0.0533	3.09	0.1410	not significant
Pure Error	0.0689	4	0.0172			
Cor Total	8.39	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 38.36 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 3.09 implies the Lack of Fit is not significant relative to the pure error. There is a 14.10% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.2174	**R^2**	0.8647
Mean	1.03	**Adjusted R^2**	0.8422
C.V. %	21.01	**Predicted R^2**	0.7951
		Adeq Precision	20.3620

The **Predicted R^2** of 0.7951 is in reasonable agreement with the **Adjusted R^2** of 0.8422; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 20.362 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.03	1	0.0404	0.9515	1.12	
A-Glucose	-0.6117	1	0.0628	-0.7412	-0.4821	1.0000
B-Glycerol	-0.3075	1	0.0628	-0.4370	-0.1780	1.0000
C-Trehalose	-0.2367	1	0.0628	-0.3662	-0.1071	1.0000
D-BSA	-0.2825	1	0.0628	-0.4120	-0.1530	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

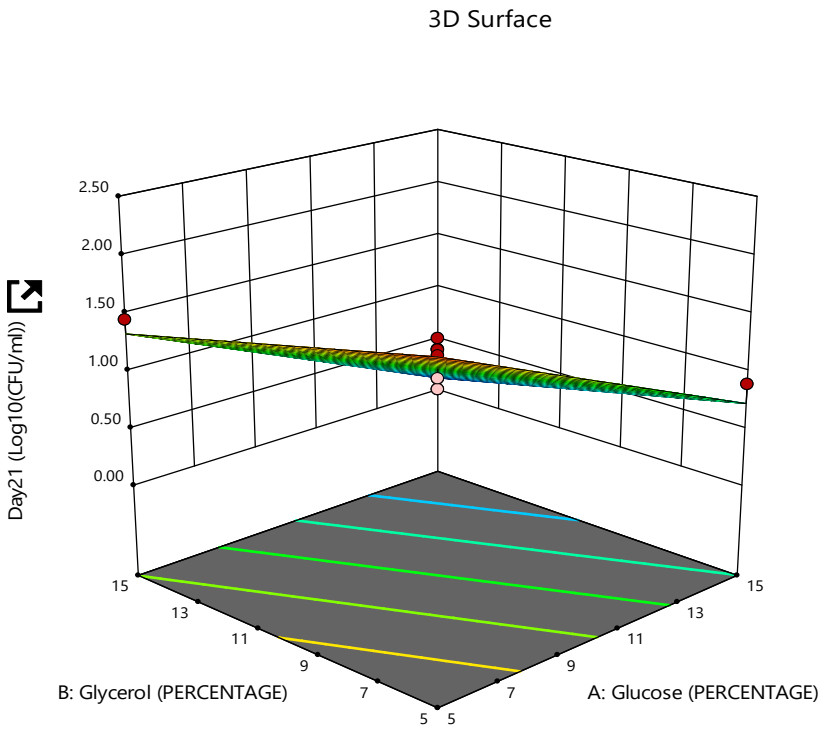
Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.13	1.93	0.2010	0.201	1.034	1.036	0.054	0.520	9
2	0.6000	0.1865	0.4135	0.201	2.128	2.313	0.228	1.160	20
3	1.08	1.03	0.0452	0.034	0.211	0.207	0.000	0.039	25
4	0.9000	0.7057	0.1943	0.201	1.000	1.000	0.050	0.502	10
5	1.34	1.55	-0.2140	0.201	-1.101	-1.106	0.061	-0.555	5
6	1.73	1.88	-0.1532	0.201	-0.788	-0.782	0.031	-0.392	17
7	0.4800	0.4907	-0.0107	0.201	-0.055	-0.054	0.000	-0.027	16
8	1.30	1.03	0.2652	0.034	1.241	1.256	0.011	0.237	26
9	0.9000	1.01	-0.1098	0.201	-0.565	-0.557	0.016	-0.279	22
10	1.15	0.9890	0.1610	0.201	0.828	0.823	0.035	0.413	7
11	0.7000	0.6598	0.0402	0.201	0.207	0.203	0.002	0.102	18
12	0.9500	1.03	-0.0848	0.034	-0.397	-0.390	0.001	-0.074	29
13	1.08	1.11	-0.0257	0.201	-0.132	-0.129	0.001	-0.065	15
14	2.01	1.95	0.0560	0.201	0.288	0.283	0.004	0.142	1
15	1.46	1.34	0.1210	0.201	0.623	0.615	0.020	0.308	3
16	1.40	1.62	-0.2248	0.201	-1.157	-1.166	0.067	-0.585	21
17	0.3000	0.4448	-0.1448	0.201	-0.745	-0.738	0.028	-0.370	24
18	1.04	1.06	-0.0198	0.201	-0.102	-0.100	0.001	-0.050	23
19	1.62	1.41	0.2102	0.201	1.081	1.086	0.059	0.545	19
20	0.7000	1.08	-0.3807	0.201	-1.959	-2.092	0.193	-1.050	6
21	1.49	1.36	0.1260	0.201	0.648	0.640	0.021	0.321	11
22	0.0000	0.5157	-0.5157	0.201	-2.653	-3.090	0.355	-1.551[(1)]	8
23	1.40	1.58	-0.1790	0.201	-0.921	-0.918	0.043	-0.461	13
24	0.0000	0.1407	-0.1407	0.201	-0.724	-0.716	0.026	-0.360	12
25	0.9000	0.7307	0.1693	0.201	0.871	0.867	0.038	0.435	2
26	1.0000	0.9640	0.0360	0.201	0.185	0.182	0.002	0.091	14
27	1.20	1.03	0.1652	0.034	0.773	0.766	0.004	0.145	27
28	0.0000	0.1157	-0.1157	0.201	-0.595	-0.587	0.018	-0.295	4
29	1.15	1.03	0.1152	0.034	0.539	0.531	0.002	0.100	28

(1) Exceeds limits.

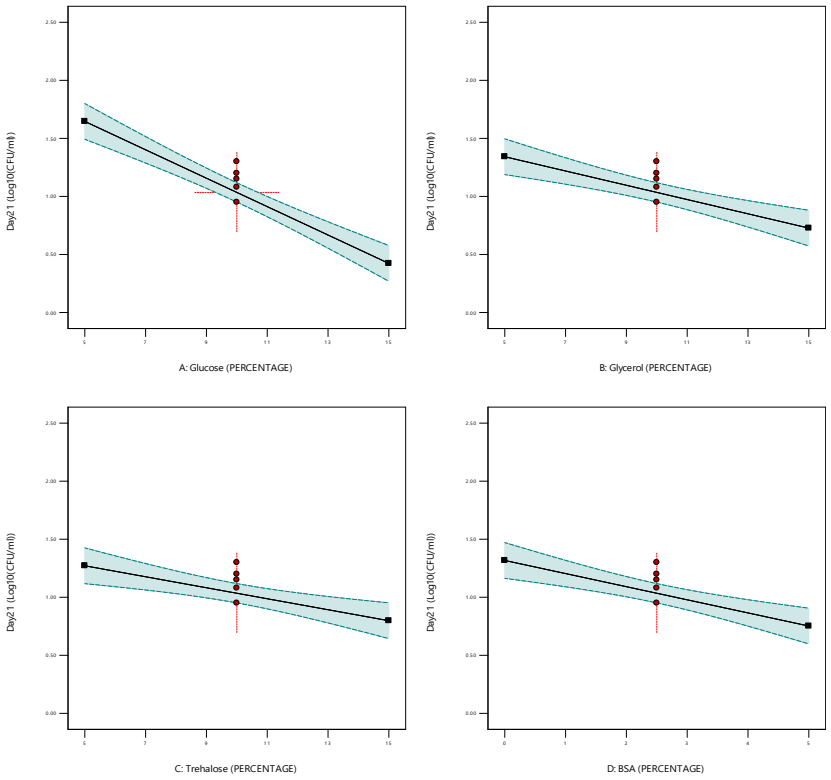
Factor Coding: Actual
Response: Day21 (Log10(CFU/ml))
Design Points:
Above Surface
Below Surface
0.00 2.13

Actual Factors:
C = 10
D = 2.5



Factor Coding: Actual
Response: Day21 (Log10(CFU/ml))
Design Points
95% CI Bands

Actual Factors:
A = 10
B = 10
C = 10
D = 2.5



Fit Summary

Response 5: Day 28

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001	0.0844	0.8738	0.8468	
2FI	0.9222	0.0561	0.8476	0.7490	
Quadratic	**0.0392**	**0.1088**	**0.9003**	**0.7332**	**Suggested**
Cubic	0.1883	0.1379	0.9391	-0.1883	**Aliased**

Sequential Model Sum of Squares [Type I]

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	29.52	1	29.52			
Linear vs Mean	8.01	4	2.00	49.48	< 0.0001	
2FI vs Linear	0.0916	6	0.0153	0.3124	0.9222	
Quadratic vs 2FI	**0.4322**	**4**	**0.1081**	**3.38**	**0.0392**	**Suggested**
Cubic vs Quadratic	0.3306	8	0.0413	2.12	0.1883	Aliased
Residual	0.1172	6	0.0195			
Total	38.51	29	1.33			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	0.2012	0.8918	0.8738	0.8468	1.38	
2FI	0.2211	0.9020	0.8476	0.7490	2.25	
Quadratic	**0.1788**	**0.9502**	**0.9003**	**0.7332**	**2.40**	**Suggested**
Cubic	0.1397	0.9870	0.9391	-0.1883	10.67	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

ANOVA for Quadratic model

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.54	14	0.6097	19.06	< 0.0001	significant
A-Glucose	5.87	1	5.87	183.40	< 0.0001	
B-Glycerol	1.09	1	1.09	34.14	< 0.0001	
C-Trehalose	0.7957	1	0.7957	24.88	0.0002	
D-BSA	0.2581	1	0.2581	8.07	0.0131	
AB	0.0072	1	0.0072	0.2259	0.6419	
AC	0.0182	1	0.0182	0.5698	0.4628	
AD	0.0306	1	0.0306	0.9575	0.3444	
BC	0.0002	1	0.0002	0.0070	0.9343	
BD	0.0081	1	0.0081	0.2533	0.6226	
CD	0.0272	1	0.0272	0.8512	0.3718	
A^2	0.0081	1	0.0081	0.2532	0.6227	
B^2	0.1590	1	0.1590	4.97	0.0426	
C^2	0.2802	1	0.2802	8.76	0.0103	
D^2	0.1347	1	0.1347	4.21	0.0594	
Residual	0.4478	14	0.0320			
Lack of Fit	0.4043	10	0.0404	3.72	0.1088	not significant
Pure Error	0.0435	4	0.0109			
Cor Total	8.98	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 19.06 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 3.72 implies the Lack of Fit is not significant relative to the pure error. There is a 10.88% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1788	**R²**	0.9502
Mean	1.01	**Adjusted R²**	0.9003
C.V. %	17.73	**Predicted R²**	0.7332
		Adeq Precision	16.4727

The **Predicted R²** of 0.7332 is in reasonable agreement with the **Adjusted R²** of 0.9003; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 16.473 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.23	1	0.0800	1.06	1.41	
A-Glucose	-0.6992	1	0.0516	-0.8099	-0.5884	1.0000
B-Glycerol	-0.3017	1	0.0516	-0.4124	-0.1909	1.0000
C-Trehalose	-0.2575	1	0.0516	-0.3682	-0.1468	1.0000
D-BSA	-0.1467	1	0.0516	-0.2574	-0.0359	1.0000
AB	0.0425	1	0.0894	-0.1493	0.2343	1.0000
AC	-0.0675	1	0.0894	-0.2593	0.1243	1.0000
AD	0.0875	1	0.0894	-0.1043	0.2793	1.0000
BC	0.0075	1	0.0894	-0.1843	0.1993	1.0000
BD	-0.0450	1	0.0894	-0.2368	0.1468	1.0000
CD	-0.0825	1	0.0894	-0.2743	0.1093	1.0000
A^2	-0.0353	1	0.0702	-0.1859	0.1153	1.08
B^2	-0.1566	1	0.0702	-0.3072	-0.0060	1.08
C^2	-0.2078	1	0.0702	-0.3584	-0.0572	1.08
D^2	-0.1441	1	0.0702	-0.2947	0.0065	1.08

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the

VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.06	1.99	0.0721	0.583	0.624	0.610	0.036	0.722	9
2	0.0000	-0.0333	0.0333	0.583	0.289	0.279	0.008	0.330	20
3	1.20	1.23	-0.0340	0.200	-0.213	-0.205	0.001	-0.103	25
4	0.4800	0.4146	0.0654	0.583	0.567	0.552	0.030	0.654	10
5	1.38	1.20	0.1762	0.583	1.527	1.611	0.218	1.907	5
6	1.69	1.88	-0.1900	0.583	-1.646	-1.766	0.253	-2.090	17
7	0.3000	0.3179	-0.0179	0.583	-0.155	-0.150	0.002	-0.177	16
8	1.34	1.23	0.1060	0.200	0.663	0.649	0.007	0.324	26
9	0.6000	0.8233	-0.2233	0.583	-1.935	-2.178	0.349	-2.577(1)	22
10	1.23	1.08	0.1546	0.583	1.339	1.382	0.167	1.635	7
11	0.3000	0.6167	-0.3167	0.583	-2.743	-3.887	0.702	-4.599(1)	18
12	1.08	1.23	-0.1540	0.200	-0.963	-0.960	0.015	-0.480	29
13	0.9000	0.9063	-0.0063	0.583	-0.054	-0.052	0.000	-0.062	15
14	1.95	2.09	-0.1354	0.583	-1.173	-1.190	0.128	-1.408	1
15	1.48	1.40	0.0829	0.583	0.718	0.705	0.048	0.834	3
16	1.32	1.34	-0.0167	0.583	-0.144	-0.139	0.002	-0.165	21
17	0.3000	0.4400	-0.1400	0.583	-1.213	-1.235	0.137	-1.462	24
18	1.20	1.13	0.0667	0.583	0.577	0.563	0.031	0.666	23
19	1.66	1.50	0.1600	0.583	1.386	1.438	0.179	1.701	19
20	0.7800	0.8538	-0.0738	0.583	-0.639	-0.625	0.038	-0.739	6
21	1.53	1.52	0.0104	0.583	0.090	0.087	0.001	0.103	11
22	0.3000	0.3954	-0.0954	0.583	-0.827	-0.817	0.064	-0.966	8
23	1.53	1.44	0.0937	0.583	0.812	0.802	0.062	0.949	13
24	0.3000	0.2962	0.0038	0.583	0.032	0.031	0.000	0.037	12
25	0.6000	0.6021	-0.0021	0.583	-0.018	-0.017	0.000	-0.021	2
26	0.9000	0.8179	0.0821	0.583	0.711	0.698	0.047	0.826	14
27	1.32	1.23	0.0860	0.200	0.538	0.524	0.005	0.262	27
28	0.3000	0.0838	0.2162	0.583	1.873	2.085	0.328	2.467(1)	4
29	1.23	1.23	-0.0040	0.200	-0.025	-0.024	0.000	-0.012	28

(1) Exceeds limits.

Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points:

Above Surface

Below Surface

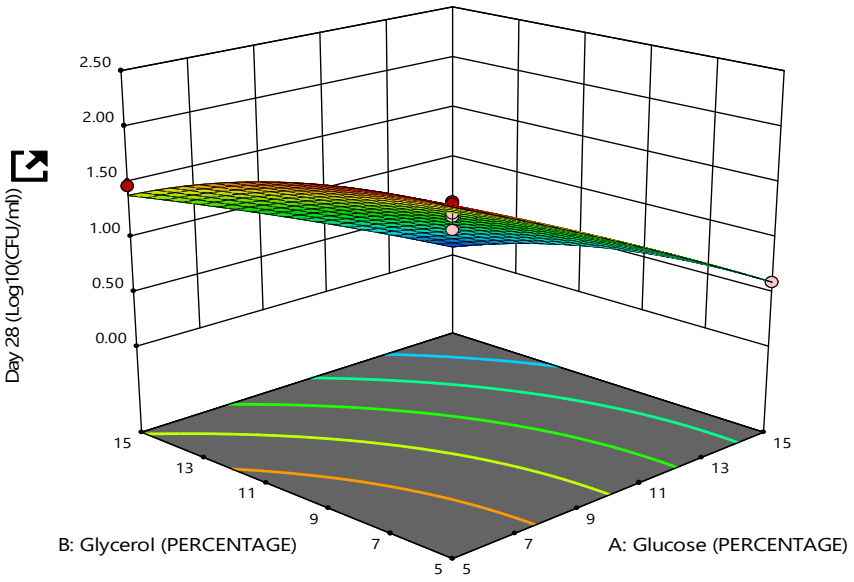
0.00 2.06

Actual Factors:

C = 10

D = 2.5

3D Surface



Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points

95% CI Bands

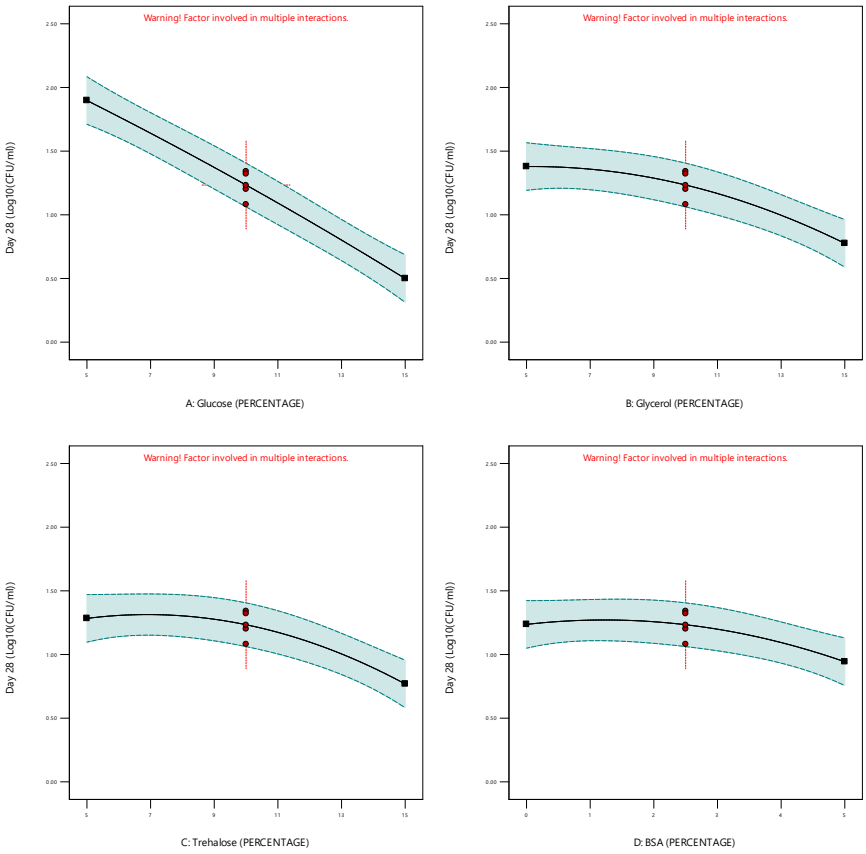
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Appendix 5: Raw data from RSM software for protectants study of *E. coli*

Build Information

File Version	22.0.0.0		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	29
Design Model	Quadratic	**Blocks**	No Blocks

Responses

Response	Name	Units	Observations	Minimum	Maximum	Mean	Std. Dev.	Ratio
R1	Day 0	Log10(CFU/ml)	29.00	1.48	2.36	1.91	0.2182	1.59
R2	Day 7	Log10 (cFU/ml)	29.00	1.45	2.38	1.91	0.2243	1.64
R3	Day 14	Log10 (CFU/ml)	29.00	1.26	2.34	1.88	0.2397	1.86
R4	Day21	Log10(CFU/ml)	29.00	0.95	2.40	1.78	0.2922	2.53
R5	Day 28	Log10(CFU/ml)	29.00	0.60	2.33	1.56	0.3951	3.88

ANOVA for Linear model

Response 1: Day 0

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.31	4	0.3281	385.18	< 0.0001	significant
A-Glucose	1.04	1	1.04	1218.98	< 0.0001	
B-Glycerol	0.0001	1	0.0001	0.0880	0.7692	
C-Trehalose	0.0070	1	0.0070	8.23	0.0085	
D-BSA	0.2670	1	0.2670	313.44	< 0.0001	
Residual	0.0204	24	0.0009			
Lack of Fit	0.0151	20	0.0008	0.5686	0.8231	not significant
Pure Error	0.0053	4	0.0013			
Cor Total	1.33	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 385.18 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.57 implies the Lack of Fit is not significant relative to the pure error. There is a 82.31% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.0292	**R^2**	0.9847
Mean	1.91	**Adjusted R^2**	0.9821
C.V. %	1.53	**Predicted R^2**	0.9780
		Adeq Precision	73.1625

The **Predicted R^2** of 0.9780 is in reasonable agreement with the **Adjusted R^2** of 0.9821; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 73.162 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.91	1	0.0054	1.90	1.92	
A-Glucose	-0.2942	1	0.0084	-0.3116	-0.2768	1.0000
B-Glycerol	-0.0025	1	0.0084	-0.0199	0.0149	1.0000
C-Trehalose	0.0242	1	0.0084	0.0068	0.0416	1.0000
D-BSA	-0.1492	1	0.0084	-0.1666	-0.1318	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.36	2.35	0.0053	0.201	0.203	0.199	0.002	0.100	9
2	1.60	1.64	-0.0414	0.201	-1.586	-1.641	0.127	-0.824	20
3	1.98	1.91	0.0686	0.034	2.393	2.684	0.041	0.507	25
4	1.81	1.77	0.0436	0.201	1.672	1.742	0.141	0.874	10
5	2.02	2.04	-0.0164	0.201	-0.628	-0.620	0.020	-0.311	5
6	2.14	2.18	-0.0414	0.201	-1.586	-1.641	0.127	-0.824	17
7	1.96	1.93	0.0270	0.201	1.033	1.035	0.054	0.519	16
8	1.89	1.91	-0.0214	0.034	-0.745	-0.738	0.004	-0.140	26
9	2.04	2.06	-0.0180	0.201	-0.692	-0.684	0.024	-0.343	22
10	1.75	1.74	0.0120	0.201	0.458	0.451	0.011	0.226	7
11	1.57	1.59	-0.0230	0.201	-0.883	-0.879	0.039	-0.441	18
12	1.91	1.91	-0.0014	0.034	-0.048	-0.047	0.000	-0.009	29
13	1.94	1.94	0.0020	0.201	0.075	0.073	0.000	0.037	15
14	2.24	2.21	0.0320	0.201	1.225	1.238	0.076	0.621	1
15	2.23	2.20	0.0270	0.201	1.033	1.035	0.054	0.519	3
16	2.02	2.06	-0.0430	0.201	-1.650	-1.716	0.137	-0.861	21
17	1.72	1.76	-0.0397	0.201	-1.522	-1.568	0.117	-0.787	24
18	1.74	1.76	-0.0247	0.201	-0.947	-0.945	0.045	-0.474	23
19	2.22	2.23	-0.0097	0.201	-0.372	-0.366	0.007	-0.183	19
20	2.08	2.08	-0.0047	0.201	-0.181	-0.177	0.002	-0.089	6
21	2.06	2.06	0.0036	0.201	0.139	0.136	0.001	0.068	11
22	1.79	1.79	0.0036	0.201	0.139	0.136	0.001	0.068	8
23	1.92	1.89	0.0303	0.201	1.161	1.170	0.068	0.587	13
24	1.48	1.47	0.0120	0.201	0.458	0.451	0.011	0.226	12
25	1.64	1.62	0.0203	0.201	0.778	0.771	0.030	0.387	2
26	1.90	1.88	0.0153	0.201	0.586	0.578	0.017	0.290	14
27	1.90	1.91	-0.0114	0.034	-0.397	-0.390	0.001	-0.074	27
28	1.62	1.61	0.0053	0.201	0.203	0.199	0.002	0.100	4
29	1.90	1.91	-0.0114	0.034	-0.397	-0.390	0.001	-0.074	28

Fit Summary

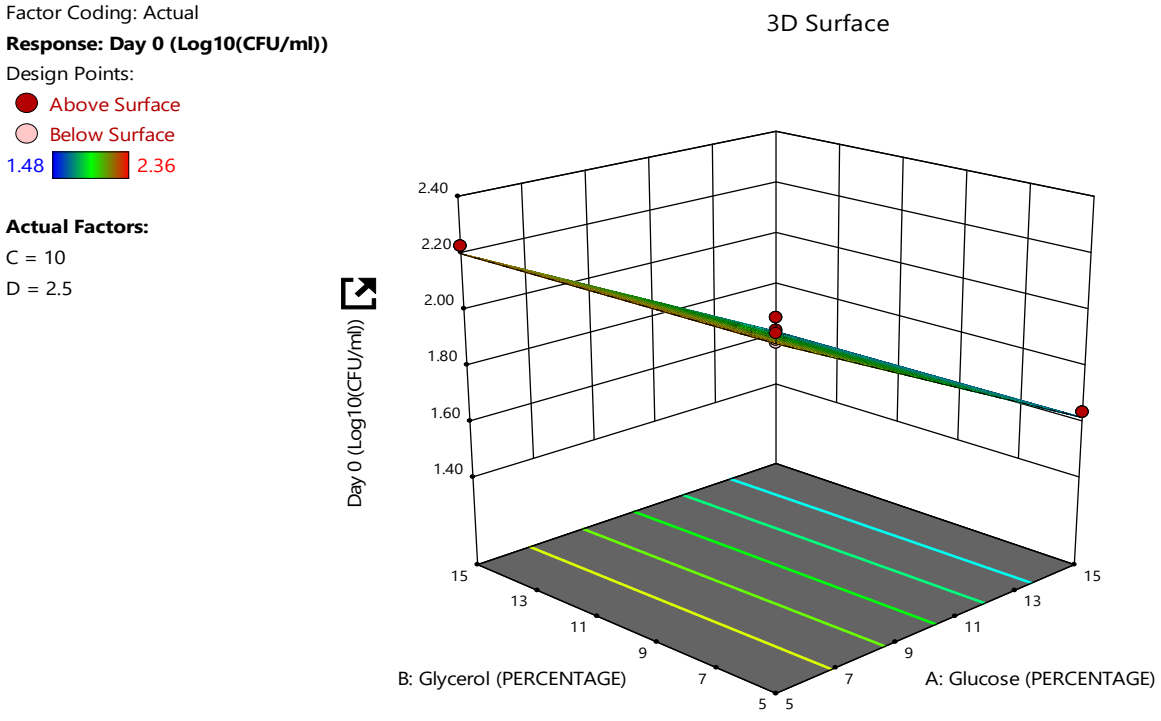
Response 1: Day 0

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.8231**	**0.9821**	**0.9780**	**Suggested**
2FI	0.9364	0.7138	0.9782	0.9627	
Quadratic	0.9150	0.5883	0.9737	0.9411	
Cubic	0.9987	0.1140	0.9448	-0.1334	**Aliased**

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.0292**	**0.9847**	**0.9821**	**0.9780**	**0.0294**	**Suggested**
2FI	0.0322	0.9860	0.9782	0.9627	0.0498	
Quadratic	0.0354	0.9869	0.9737	0.9411	0.0785	
Cubic	0.0512	0.9882	0.9448	-0.1334	1.51	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.



Factor Coding: Actual

Response: Day 0 (Log10(CFU/ml))

Design Points

95% CI Bands

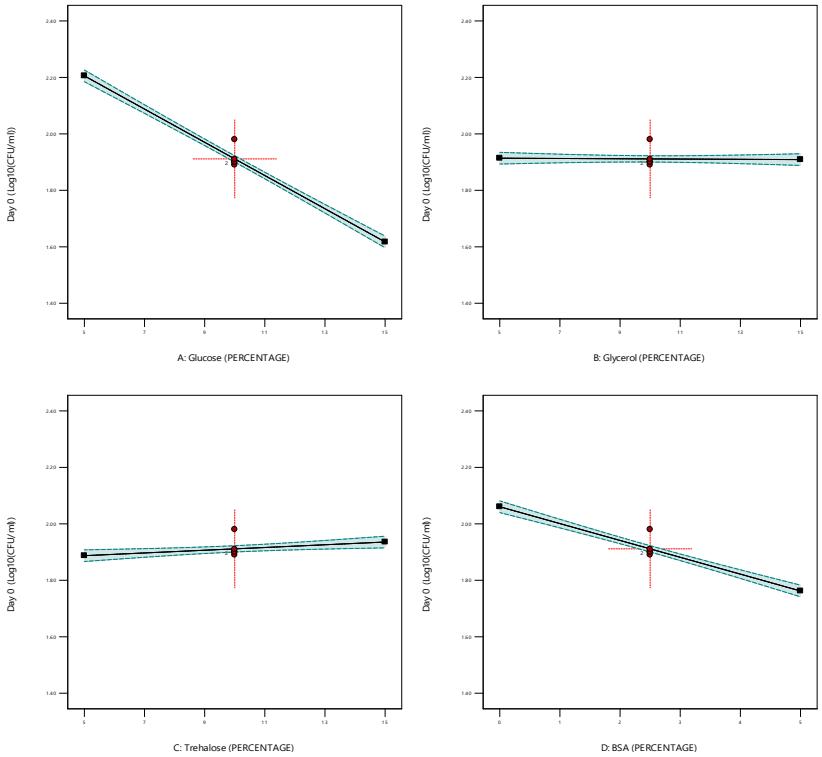
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 2: Day 7

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.9332**	**0.9397**	**0.9278**	**Suggested**
2FI	0.9843	0.8462	0.9237	0.8770	
Quadratic	0.8499	0.7593	0.9105	0.8164	
Cubic	0.9765	0.2561	0.8368	-1.5145	**Aliased**

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.0551**	**0.9483**	**0.9397**	**0.9278**	**0.1017**	**Suggested**

2FI	0.0619	0.9510	0.9237	0.8770	0.1733	
Quadratic	0.0671	0.9552	0.9105	0.8164	0.2585	
Cubic	0.0906	0.9650	0.8368	-1.5145	3.54	Aliased

Focus on the model maximizing the **Adjusted R²** and the **Predicted R²**.

ANOVA for Linear model

Response 2: Day 7

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.34	4	0.3339	110.16	< 0.0001	significant
A-Glucose	1.00	1	1.00	331.01	< 0.0001	
B-Glycerol	0.0003	1	0.0003	0.0990	0.7558	
C-Trehalose	0.0184	1	0.0184	6.07	0.0213	
D-BSA	0.3136	1	0.3136	103.46	< 0.0001	
Residual	0.0728	24	0.0030			
Lack of Fit	0.0478	20	0.0024	0.3839	0.9332	not significant
Pure Error	0.0249	4	0.0062			
Cor Total	1.41	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 110.16 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.38 implies the Lack of Fit is not significant relative to the pure error. There is a 93.32% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.91	1	0.0102	1.88	1.93	
A-Glucose	-0.2892	1	0.0159	-0.3220	-0.2564	1.0000
B-Glycerol	0.0050	1	0.0159	-0.0278	0.0378	1.0000
C-Trehalose	0.0392	1	0.0159	0.0064	0.0720	1.0000
D-BSA	-0.1617	1	0.0159	-0.1945	-0.1289	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Fit Statistics

Std. Dev.	0.0551	**R^2**	0.9483
Mean	1.91	**Adjusted R^2**	0.9397
C.V. %	2.89	**Predicted R^2**	0.9278
		Adeq Precision	39.4402

The **Predicted R^2** of 0.9278 is in reasonable agreement with the **Adjusted R^2** of 0.9397; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 39.440 indicates an adequate signal. This model can be used to navigate the design space.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.38	2.36	0.0233	0.201	0.474	0.466	0.011	0.234	9
2	1.62	1.66	-0.0359	0.201	-0.729	-0.721	0.027	-0.362	20
3	2.02	1.91	0.1141	0.034	2.110	2.288	0.032	0.432	25
4	1.83	1.78	0.0516	0.201	1.049	1.052	0.055	0.528	10
5	1.99	2.03	-0.0384	0.201	-0.780	-0.773	0.031	-0.388	5
6	2.15	2.16	-0.0059	0.201	-0.119	-0.117	0.001	-0.059	17
7	1.93	1.95	-0.0200	0.201	-0.407	-0.400	0.008	-0.201	16
8	1.83	1.91	-0.0759	0.034	-1.402	-1.433	0.014	-0.271	26
9	2.01	2.07	-0.0625	0.201	-1.271	-1.288	0.081	-0.646	22
10	1.69	1.71	-0.0150	0.201	-0.305	-0.300	0.005	-0.150	7
11	1.54	1.58	-0.0375	0.201	-0.763	-0.756	0.029	-0.379	18
12	1.92	1.91	0.0141	0.034	0.261	0.256	0.000	0.048	29
13	1.96	1.94	0.0200	0.201	0.406	0.399	0.008	0.200	15
14	2.20	2.19	0.0100	0.201	0.203	0.199	0.002	0.100	1
15	2.25	2.20	0.0500	0.201	1.015	1.016	0.052	0.510	3
16	2.01	2.06	-0.0525	0.201	-1.067	-1.071	0.057	-0.537	21
17	1.68	1.75	-0.0692	0.201	-1.406	-1.437	0.100	-0.721	24
18	1.69	1.74	-0.0492	0.201	-1.000	-1.000	0.050	-0.502	23
19	2.19	2.23	-0.0442	0.201	-0.898	-0.894	0.041	-0.449	19
20	2.13	2.11	0.0233	0.201	0.474	0.466	0.011	0.234	6
21	2.09	2.03	0.0566	0.201	1.151	1.159	0.067	0.582	11
22	1.81	1.78	0.0266	0.201	0.541	0.533	0.015	0.268	8
23	1.93	1.86	0.0683	0.201	1.388	1.417	0.097	0.711	13
24	1.45	1.46	-0.0050	0.201	-0.102	-0.100	0.001	-0.050	12
25	1.62	1.61	0.0083	0.201	0.169	0.165	0.001	0.083	2
26	1.87	1.87	-0.0017	0.201	-0.034	-0.034	0.000	-0.017	14
27	1.83	1.91	-0.0759	0.034	-1.402	-1.433	0.014	-0.271	27
28	1.73	1.62	0.1083	0.201	2.201	2.412	0.244	1.210	4
29	1.92	1.91	0.0141	0.034	0.261	0.256	0.000	0.048	28

Factor Coding: Actual

Response: Day 7 (Log10 (cFU/ml))

Design Points:

Above Surface

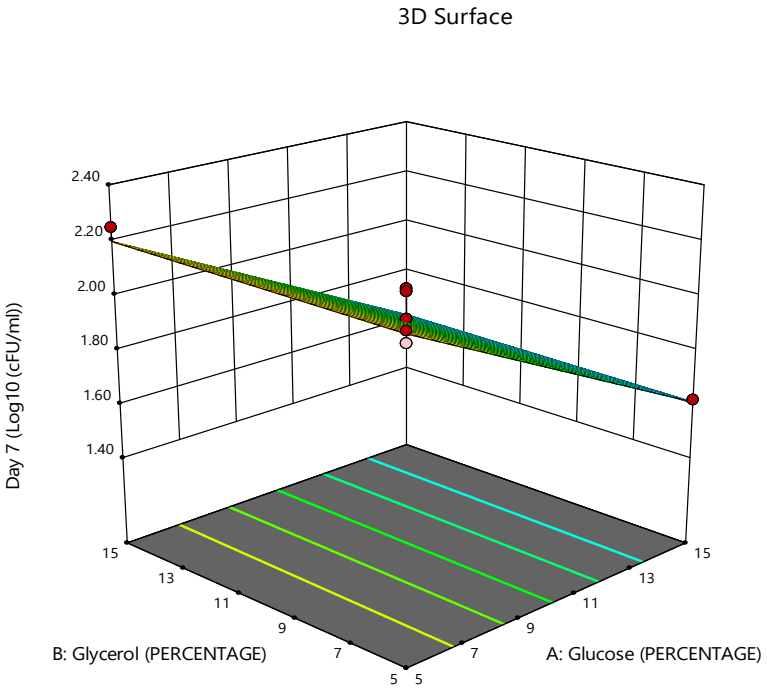
Below Surface

1.45 2.38

Actual Factors:

C = 10

D = 2.5



Factor Coding: Actual

Response: Day 7 (Log10 (cFU/ml))

Design Points

95% CI Bands

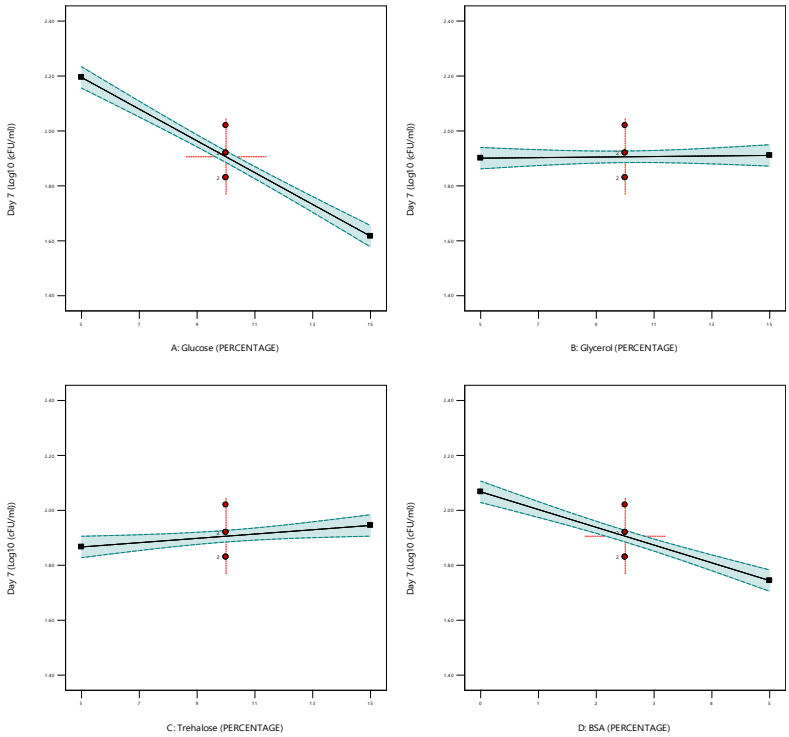
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 3: Day 14

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.4213**	**0.8998**	**0.8710**	**Suggested**
2FI	0.5377	0.3909	0.8963	0.8042	
Quadratic	0.6708	0.3285	0.8861	0.7183	
Cubic	0.2860	0.3746	0.9160	-0.0226	**Aliased**

Sequential Model Sum of Squares [Type I]

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	102.95	1	102.95			
Linear vs Mean	**1.47**	**4**	**0.3677**	**63.85**	**< 0.0001**	**Suggested**
2FI vs Linear	0.0310	6	0.0052	0.8666	0.5377	
Quadratic vs 2FI	0.0156	4	0.0039	0.5970	0.6708	
Cubic vs Quadratic	0.0626	8	0.0078	1.62	0.2860	Aliased
Residual	0.0290	6	0.0048			
Total	104.56	29	3.61			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.0759**	**0.9141**	**0.8998**	**0.8710**	**0.2076**	**Suggested**
2FI	0.0772	0.9334	0.8963	0.8042	0.3151	
Quadratic	0.0809	0.9431	0.8861	0.7183	0.4533	
Cubic	0.0695	0.9820	0.9160	-0.0226	1.65	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

ANOVA for Linear model

Response 3: Day 14

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.47	4	0.3677	63.85	< 0.0001	significant
A-Glucose	1.10	1	1.10	191.74	< 0.0001	
B-Glycerol	0.0176	1	0.0176	3.06	0.0929	
C-Trehalose	0.0021	1	0.0021	0.3705	0.5485	
D-BSA	0.3468	1	0.3468	60.22	< 0.0001	
Residual	0.1382	24	0.0058			
Lack of Fit	0.1205	20	0.0060	1.36	0.4213	not significant
Pure Error	0.0177	4	0.0044			
Cor Total	1.61	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 63.85 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.36 implies the Lack of Fit is not significant relative to the pure error. There is a 42.13% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.0759	**R^2**	0.9141
Mean	1.88	**Adjusted R^2**	0.8998
C.V. %	4.03	**Predicted R^2**	0.8710
		Adeq Precision	30.0440

The **Predicted R^2** of 0.8710 is in reasonable agreement with the **Adjusted R^2** of 0.8998; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 30.044 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.88	1	0.0141	1.86	1.91	
A-Glucose	-0.3033	1	0.0219	-0.3485	-0.2581	1.0000
B-Glycerol	0.0383	1	0.0219	-0.0069	0.0835	1.0000
C-Trehalose	0.0133	1	0.0219	-0.0319	0.0585	1.0000
D-BSA	-0.1700	1	0.0219	-0.2152	-0.1248	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.34	2.36	-0.0175	0.201	-0.258	-0.253	0.003	-0.127	9
2	1.56	1.59	-0.0341	0.201	-0.503	-0.495	0.013	-0.249	20
3	1.99	1.88	0.1059	0.034	1.420	1.452	0.014	0.274	25
4	1.80	1.75	0.0492	0.201	0.725	0.718	0.026	0.360	10
5	2.01	2.04	-0.0308	0.201	-0.454	-0.447	0.010	-0.224	5
6	2.13	2.17	-0.0441	0.201	-0.651	-0.643	0.021	-0.323	17
7	1.88	1.94	-0.0558	0.201	-0.823	-0.817	0.034	-0.410	16
8	1.81	1.88	-0.0741	0.034	-0.994	-0.994	0.007	-0.188	26
9	2.03	2.09	-0.0625	0.201	-0.921	-0.918	0.043	-0.461	22
10	1.67	1.70	-0.0308	0.201	-0.454	-0.447	0.010	-0.224	7
11	1.48	1.57	-0.0875	0.201	-1.290	-1.309	0.084	-0.657	18
12	1.86	1.88	-0.0241	0.034	-0.324	-0.318	0.001	-0.060	29
13	1.89	1.86	0.0309	0.201	0.455	0.447	0.010	0.224	15
14	2.14	2.15	-0.0091	0.201	-0.135	-0.132	0.001	-0.066	1

15	2.26	2.23	0.0342	0.201	0.504	0.496	0.013	0.249	3
16	2.01	2.02	-0.0058	0.201	-0.086	-0.084	0.000	-0.042	21
17	1.78	1.75	0.0275	0.201	0.406	0.399	0.008	0.200	24
18	1.72	1.68	0.0442	0.201	0.652	0.644	0.021	0.323	23
19	2.12	2.20	-0.0808	0.201	-1.191	-1.202	0.071	-0.603	19
20	2.10	2.07	0.0325	0.201	0.480	0.472	0.012	0.237	6
21	2.06	2.02	0.0425	0.201	0.627	0.619	0.020	0.311	11
22	1.76	1.73	0.0325	0.201	0.480	0.472	0.012	0.237	8
23	1.96	1.83	0.1275	0.201	1.880	1.993	0.178	1.000	13
24	1.26	1.41	-0.1508	0.201	-2.223	-2.443	0.249	-1.226	12
25	1.49	1.54	-0.0525	0.201	-0.774	-0.767	0.030	-0.385	2
26	1.90	1.91	-0.0091	0.201	-0.135	-0.132	0.001	-0.066	14
27	1.91	1.88	0.0259	0.034	0.347	0.340	0.001	0.064	27
28	1.82	1.62	0.2009	0.201	2.961	3.639(1)	0.442	1.826(2)	4
29	1.90	1.88	0.0159	0.034	0.213	0.208	0.000	0.039	28

(1) Observation with |External Stud. Residuals| > 3.55

(2) Exceeds limits.

Factor Coding: Actual

Response: Day 14 (Log10 (CFU/ml))

Design Points:

Above Surface

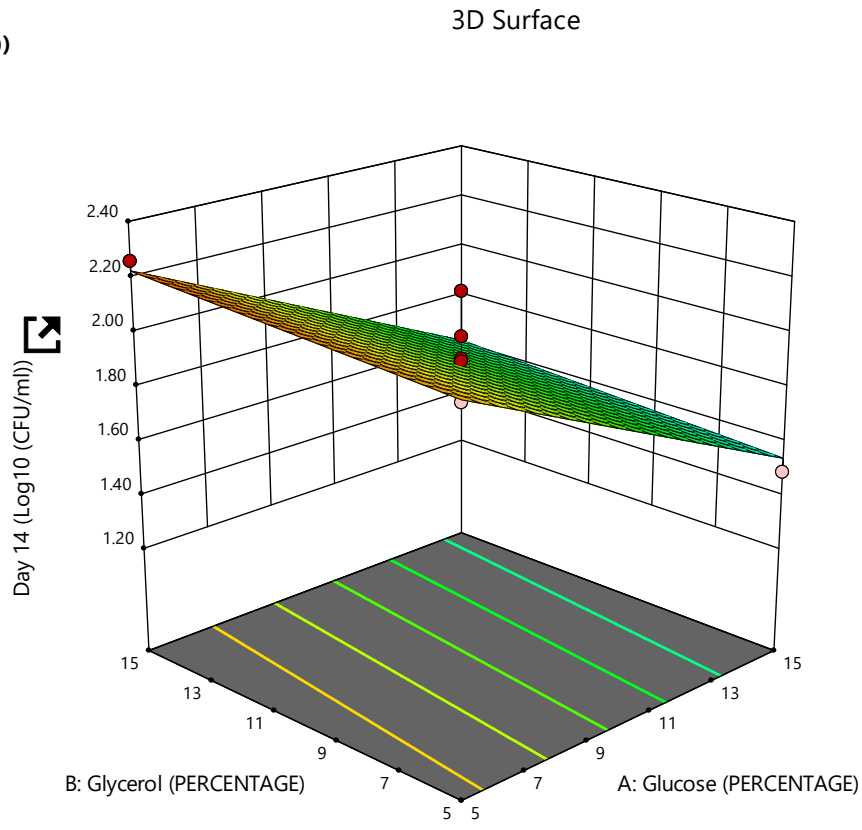
Below Surface

1.26 2.34

Actual Factors:

C = 10

D = 2.5



Factor Coding: Actual

Response: Day 14 (Log10 (CFU/ml))

Design Points

95% CI Bands

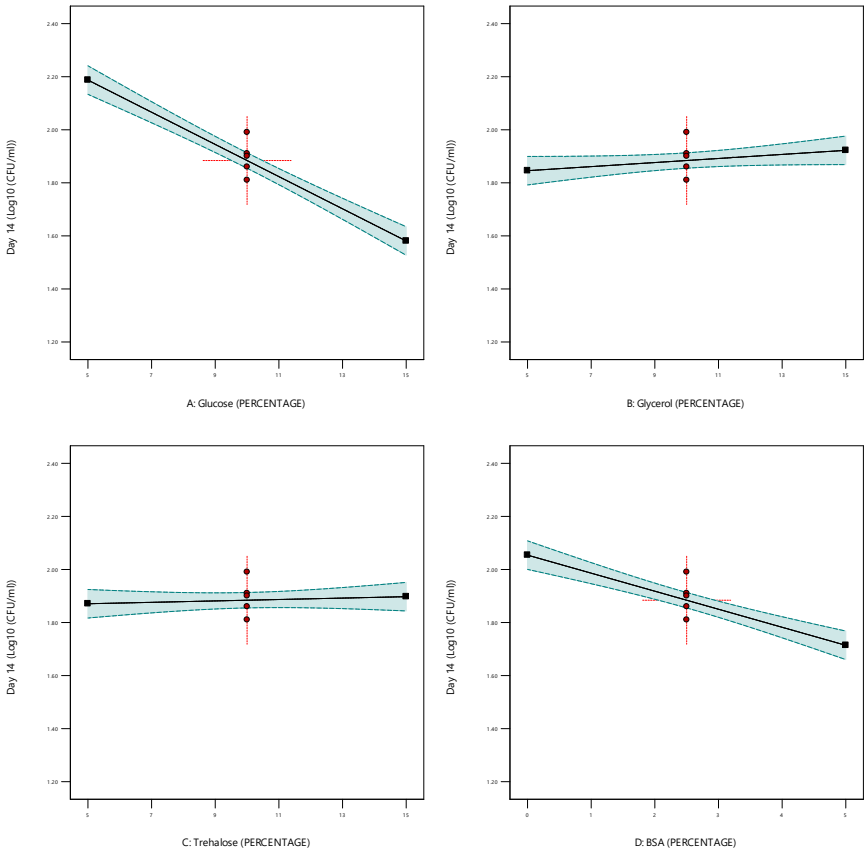
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 4: Day21

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.3590**	**0.8566**	**0.8158**	**Suggested**
2FI	0.3143	0.3796	0.8660	0.7521	
Quadratic	0.7426	0.3062	0.8490	0.6234	
Cubic	0.1075	0.7937	0.9271	0.7328	**Aliased**

Sequential Model Sum of Squares [Type I]

Response 4: Day21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	92.31	1	92.31			
Linear vs Mean	**2.10**	**4**	**0.5242**	**42.81**	**< 0.0001**	**Suggested**
2FI vs Linear	0.0880	6	0.0147	1.28	0.3143	
Quadratic vs 2FI	0.0253	4	0.0063	0.4909	0.7426	
Cubic vs Quadratic	0.1432	8	0.0179	2.88	0.1075	Aliased
Residual	0.0374	6	0.0062			
Total	94.70	29	3.27			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

ANOVA for Linear model

Response 4: Day21

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.10	4	0.5242	42.81	< 0.0001	significant
A-Glucose	1.48	1	1.48	120.63	< 0.0001	
B-Glycerol	0.0290	1	0.0290	2.37	0.1368	
C-Trehalose	0.0012	1	0.0012	0.0980	0.7569	
D-BSA	0.5896	1	0.5896	48.16	< 0.0001	
Residual	0.2939	24	0.0122			
Lack of Fit	0.2606	20	0.0130	1.57	0.3590	not significant
Pure Error	0.0333	4	0.0083			
Cor Total	2.39	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 42.81 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, D are significant model terms. Values greater than 0.1000 indicate the model terms are not

significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.57 implies the Lack of Fit is not significant relative to the pure error. There is a 35.90% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1107	**R²**	0.8771
Mean	1.78	**Adjusted R²**	0.8566
C.V. %	6.20	**Predicted R²**	0.8158
		Adeq Precision	24.9207

The **Predicted R²** of 0.8158 is in reasonable agreement with the **Adjusted R²** of 0.8566; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 24.921 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.78	1	0.0205	1.74	1.83	
A-Glucose	-0.3508	1	0.0319	-0.4168	-0.2849	1.0000
B-Glycerol	0.0492	1	0.0319	-0.0168	0.1151	1.0000
C-Trehalose	0.0100	1	0.0319	-0.0559	0.0759	1.0000
D-BSA	-0.2217	1	0.0319	-0.2876	-0.1557	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.40	2.36	0.0434	0.201	0.438	0.431	0.010	0.216	9
2	1.43	1.44	-0.0133	0.201	-0.135	-0.132	0.001	-0.066	20
3	1.89	1.78	0.1059	0.034	0.974	0.973	0.007	0.184	25
4	1.77	1.65	0.1150	0.201	1.163	1.172	0.068	0.588	10
5	1.87	2.00	-0.1258	0.201	-1.272	-1.289	0.081	-0.647	5
6	2.06	2.12	-0.0650	0.201	-0.657	-0.649	0.022	-0.326	17
7	1.83	1.84	-0.0133	0.201	-0.135	-0.132	0.001	-0.066	16
8	1.67	1.78	-0.1141	0.034	-1.050	-1.052	0.008	-0.199	26
9	1.99	2.05	-0.0650	0.201	-0.657	-0.649	0.022	-0.326	22
10	1.56	1.55	0.0075	0.201	0.076	0.075	0.000	0.037	7
11	1.41	1.42	-0.0133	0.201	-0.135	-0.132	0.001	-0.066	18
12	1.81	1.78	0.0259	0.034	0.238	0.233	0.000	0.044	29
13	1.78	1.74	0.0350	0.201	0.354	0.348	0.006	0.174	15
14	2.03	2.09	-0.0558	0.201	-0.564	-0.556	0.016	-0.279	1
15	2.16	2.18	-0.0241	0.201	-0.244	-0.239	0.003	-0.120	3
16	1.95	1.96	-0.0066	0.201	-0.067	-0.066	0.000	-0.033	21
17	1.69	1.61	0.0784	0.201	0.792	0.786	0.032	0.394	24
18	1.64	1.51	0.1267	0.201	1.281	1.299	0.083	0.652	23
19	2.07	2.14	-0.0750	0.201	-0.758	-0.751	0.029	-0.377	19
20	2.01	2.02	-0.0058	0.201	-0.059	-0.057	0.000	-0.029	6
21	1.98	1.91	0.0667	0.201	0.674	0.667	0.023	0.334	11
22	1.51	1.57	-0.0625	0.201	-0.632	-0.624	0.020	-0.313	8
23	1.88	1.72	0.1550	0.201	1.568	1.620	0.124	0.813	13
24	0.9500	1.21	-0.2616	0.201	-2.646	-3.077	0.352	-1.544[1]	12
25	1.23	1.38	-0.1541	0.201	-1.559	-1.609	0.122	-0.808	2
26	1.73	1.82	-0.0933	0.201	-0.943	-0.941	0.045	-0.472	14
27	1.89	1.78	0.1059	0.034	0.974	0.973	0.007	0.184	27
28	1.70	1.48	0.2175	0.201	2.200	2.410	0.244	1.209	4
29	1.85	1.78	0.0659	0.034	0.606	0.598	0.003	0.113	28

[1] Exceeds limits.

Factor Coding: Actual

Response: Day21 (Log10(CFU/ml))

Design Points:

Above Surface

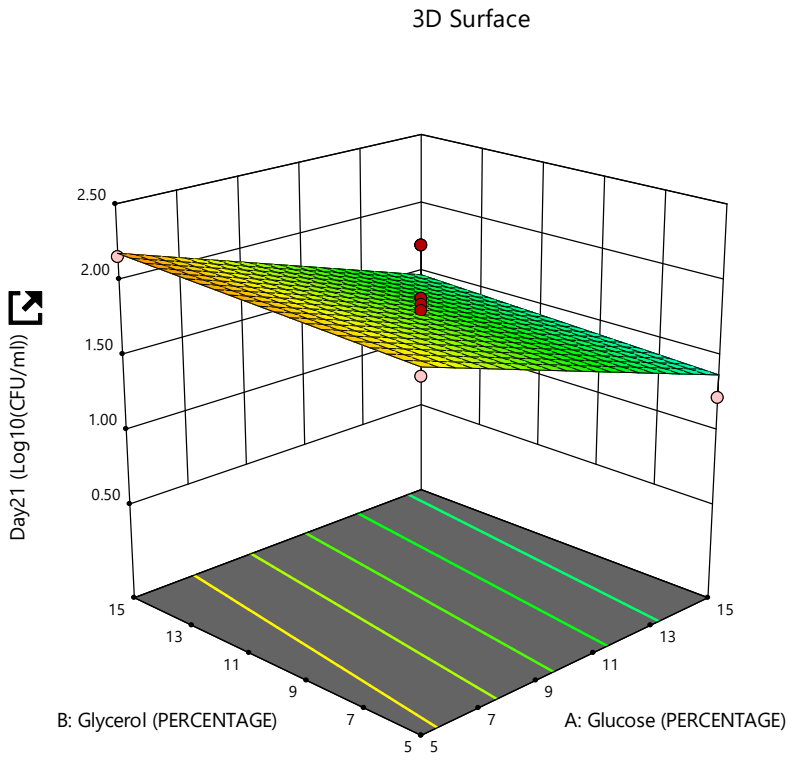
Below Surface

0.95 2.40

Actual Factors:

C = 10

D = 2.5



Factor Coding: Actual

Response: Day21 (Log10(CFU/ml))

Design Points

95% CI Bands

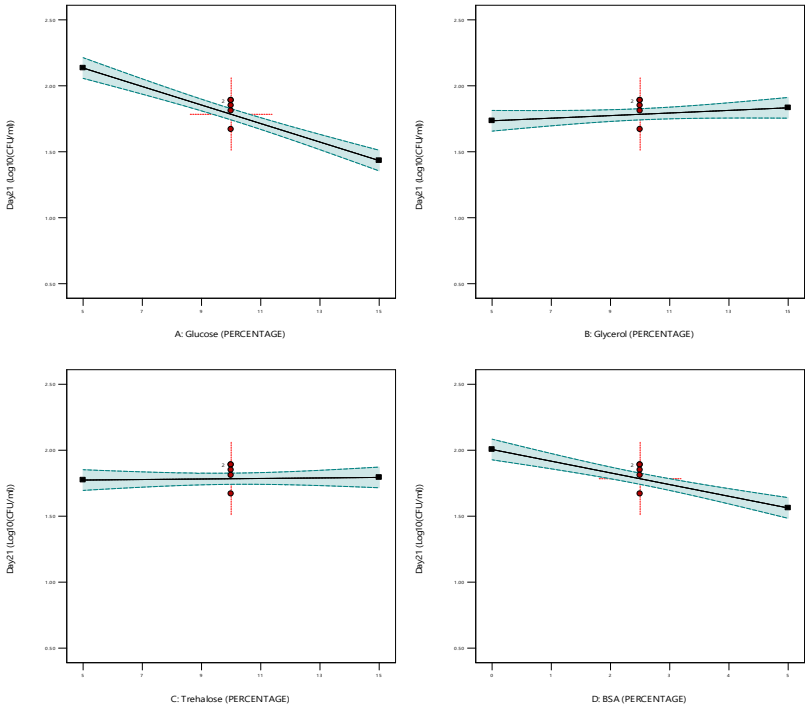
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Fit Summary

Response 5: Day 28

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.2845**	**0.8051**	**0.7490**	**Suggested**
2FI	0.1983	0.3349	0.8314	0.6889	
Quadratic	0.8238	0.2550	0.8041	0.5029	
Cubic	0.2658	0.2857	0.8604	-1.0303	**Aliased**

Sequential Model Sum of Squares [Type I]

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	70.70	1	70.70			
Linear vs Mean	**3.64**	**4**	**0.9101**	**29.92**	**< 0.0001**	**Suggested**
2FI vs Linear	0.2562	6	0.0427	1.62	0.1983	
Quadratic vs 2FI	0.0457	4	0.0114	0.3733	0.8238	
Cubic vs Quadratic	0.2974	8	0.0372	1.71	0.2658	Aliased
Residual	0.1307	6	0.0218			
Total	75.07	29	2.59			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**0.1744**	**0.8330**	**0.8051**	**0.7490**	**1.10**	**Suggested**
2FI	0.1622	0.8916	0.8314	0.6889	1.36	
Quadratic	0.1749	0.9020	0.8041	0.5029	2.17	
Cubic	0.1476	0.9701	0.8604	-1.0303	8.87	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

ANOVA for Linear model

Response 5: Day 28

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.64	4	0.9101	29.92	< 0.0001	significant
A-Glucose	2.50	1	2.50	82.29	< 0.0001	
B-Glycerol	0.0817	1	0.0817	2.69	0.1143	
C-Trehalose	0.0176	1	0.0176	0.5798	0.4538	
D-BSA	1.04	1	1.04	34.14	< 0.0001	
Residual	0.7299	24	0.0304			
Lack of Fit	0.6600	20	0.0330	1.89	0.2845	not significant
Pure Error	0.0699	4	0.0175			
Cor Total	4.37	28				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 29.92 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.89 implies the Lack of Fit is not significant relative to the pure error. There is a 28.45% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Fit Statistics

Std. Dev.	0.1744	**R^2**	0.8330
Mean	1.56	**Adjusted R^2**	0.8051
C.V. %	11.17	**Predicted R^2**	0.7490
		Adeq Precision	20.7378

The **Predicted R^2** of 0.7490 is in reasonable agreement with the **Adjusted R^2** of 0.8051; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 20.738 indicates an adequate signal. This model can be used to navigate the design space.

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.56	1	0.0324	1.49	1.63	
A-Glucose	-0.4567	1	0.0503	-0.5606	-0.3528	1.0000
B-Glycerol	0.0825	1	0.0503	-0.0214	0.1864	1.0000
C-Trehalose	0.0383	1	0.0503	-0.0656	0.1422	1.0000
D-BSA	-0.2942	1	0.0503	-0.3981	-0.1903	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.33	2.31	0.0178	0.201	0.114	0.112	0.001	0.056	9
2	1.26	1.14	0.1170	0.201	0.750	0.743	0.028	0.373	20
3	1.66	1.56	0.0986	0.034	0.576	0.567	0.002	0.107	25
4	1.53	1.40	0.1311	0.201	0.841	0.836	0.036	0.419	10
5	1.74	1.82	-0.0772	0.201	-0.495	-0.487	0.012	-0.245	5
6	1.94	1.98	-0.0397	0.201	-0.255	-0.250	0.003	-0.125	17
7	1.63	1.68	-0.0522	0.201	-0.335	-0.329	0.006	-0.165	16
8	1.41	1.56	-0.1514	0.034	-0.883	-0.879	0.006	-0.166	26
9	1.79	1.94	-0.1480	0.201	-0.950	-0.948	0.045	-0.476	22
10	1.20	1.23	-0.0289	0.201	-0.185	-0.182	0.002	-0.091	7
11	0.8500	1.07	-0.2164	0.201	-1.388	-1.417	0.097	-0.711	18
12	1.69	1.56	0.1286	0.034	0.751	0.744	0.004	0.141	29
13	1.57	1.52	0.0528	0.201	0.339	0.332	0.006	0.167	15
14	1.92	1.94	-0.0155	0.201	-0.100	-0.098	0.001	-0.049	1

15	2.08	2.10	-0.0205	0.201	-0.132	-0.129	0.001	-0.065	3
16	1.75	1.77	-0.0230	0.201	-0.148	-0.145	0.001	-0.073	21
17	1.36	1.35	0.0103	0.201	0.066	0.065	0.000	0.032	24
18	1.28	1.18	0.0953	0.201	0.611	0.603	0.019	0.303	23
19	1.97	2.06	-0.0864	0.201	-0.554	-0.546	0.015	-0.274	19
20	1.93	1.89	0.0361	0.201	0.232	0.227	0.003	0.114	6
21	1.85	1.72	0.1261	0.201	0.809	0.803	0.033	0.403	11
22	1.0000	1.31	-0.3055	0.201	-1.960	-2.094	0.194	-1.051	8
23	1.72	1.44	0.2795	0.201	1.793	1.886	0.162	0.946	13
24	0.8500	0.8105	0.0395	0.201	0.253	0.248	0.003	0.125	12
25	0.6000	1.02	-0.4222	0.201	-2.709	-3.182	0.370	-1.597[(1)]	2
26	1.45	1.61	-0.1555	0.201	-0.998	-0.998	0.050	-0.501	14
27	1.64	1.56	0.0786	0.034	0.459	0.451	0.002	0.085	27
28	1.52	1.19	0.3328	0.201	2.135	2.322	0.230	1.165	4
29	1.76	1.56	0.1986	0.034	1.159	1.168	0.010	0.221	28

[(1)] Exceeds limits.

Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points:

Above Surface

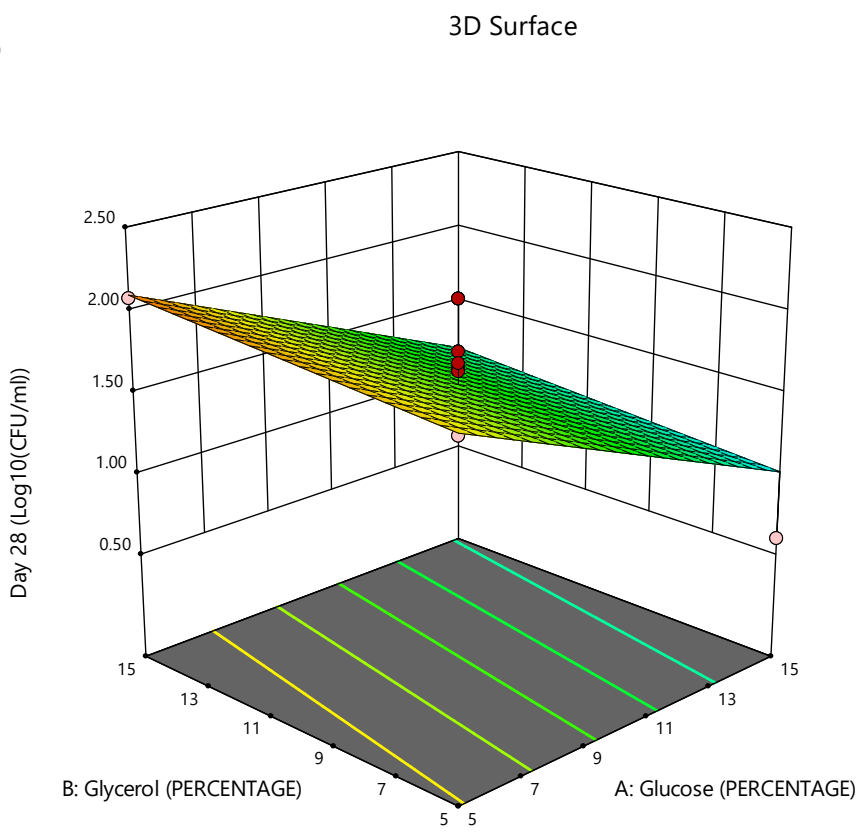
Below Surface

0.60 2.33

Actual Factors:

C = 10

D = 2.5



Factor Coding: Actual

Response: Day 28 (Log10(CFU/ml))

Design Points

95% CI Bands

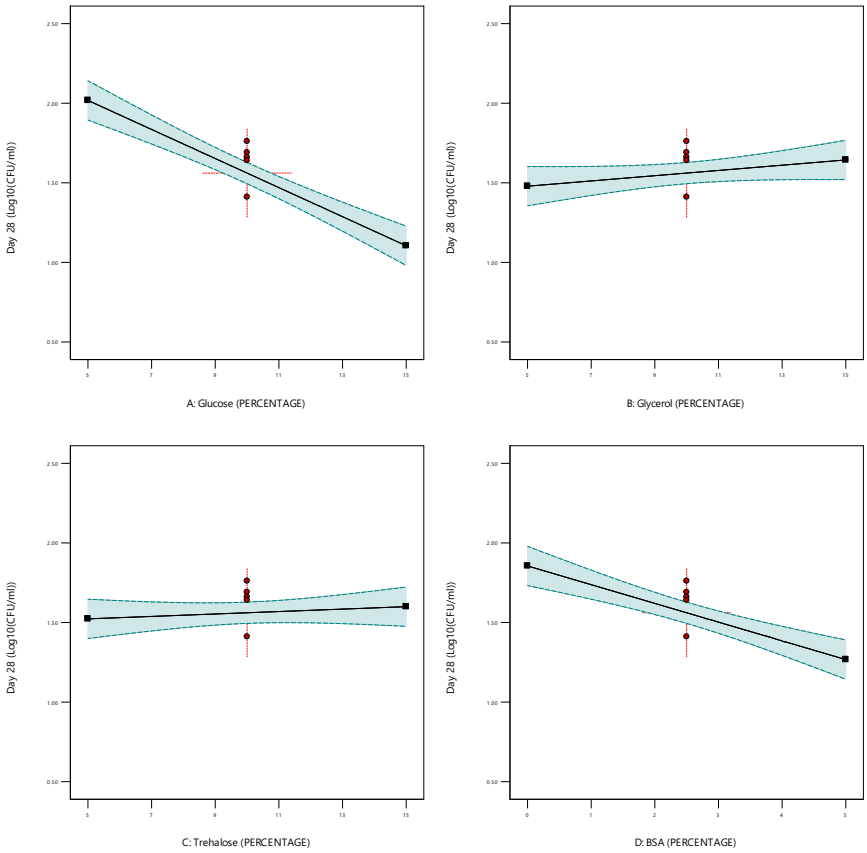
Actual Factors:

A = 10

B = 10

C = 10

D = 2.5



Appendix 6: Certificate of analysis of commercial reference material procured

Supelco®

www.sigmaaldrich.com

Certified reference material – Vitroids™
Reference material certificate

Escherichia coli WDCM 00013 VT000133

Product no.:	**VT000133**
Lot no.:	**BCCH4936**
Description of CRM:	Vitroids™ are disc-shaped, microbiological reference materials. Each disc contains a quantified number of microorganisms (colony forming units; cfu), immobilized in a solid water soluble matrix.
Expiry date:	**MAY 2024**
Storage:	**-20 ± 5 °C**; store the mylar bag containing the plastic vials with the Vitroids™ unopened
Starting material:	**CECT 434 batch 23-03-2017** (freeze-dried microorganism in a glass ampoule)
No. of passages:	**2** (upon receipt from cell culture collection CECT)

Sample: *Escherichia coli* WDCM 00013 VT000133		
Certified value (geometric mean value)	Expanded uncertainty (log_{10} value)	Expected range
9.3E+01 cfu per disc	0.029	**4.7E+01 - 1.9E+02 cfu per disc**
Conditions:	**Trypcase soy agar / aerobic / 37 °C / 24 hrs**	
Date of testing:	02 JUN 2022	

cfu: colony forming units
The reference values are calculated by (US EPA Environmental Systems Monitoring Laboratory in Cincinnati) EMSL-CIN's computer program "BIWEIGHT". The measurement of uncertainty originates from the generated biweight standard deviation (SD) resulting from the biweight geometric mean value obtained during homogeneity testing. The expected range takes into account media batch to batch variability, which is done by multiplying the biweight standard deviation by 1.6.

Metrological traceability:	Details see "Certification process details" on page 2.
Measurement method:	The certified value is established by plate counting in accordance with ISO/IEC 17025[1].
Intended, correct use & handling instructions:	Please follow the instructions given in "General instructions for intended uses of this reference material" on page 3.
Health and safety information:	Please refer to the Safety Data Sheet (link on page 3) for detailed information about the nature of any hazard and appropriate precautions to be taken.
Accreditation:	Sigma-Aldrich Production GmbH is accredited by the Swiss Accreditation Service SAS as reference material producer under no. SRMS 0001 in accordance with international standard ISO 17034 [2].
Certificate issue date:	**15 JUL 2022**

ISO 17034
SRMS 0001

Dr. Thomas Bühler – CRM Operations Dr. Philipp Zell Approving Officer

Sigma-Aldrich Production GmbH, Industriestrasse 25, 9471 Buchs, Switzerland; Tel +41-81-755-2511
www.sigmaaldrich.com
Sigma-Aldrich Production GmbH is a subsidiary of Merck KGaA, Darmstadt, Germany.

Supelco®

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Certified reference material – Vitroids™
Reference material certificate

Salmonella enterica subsp. *enterica* serovar Enteritidis WDCM 00030 VT000303

Product no.:	**VT000303**
Lot no.:	**BCCG2047**
Description of CRM:	Vitroids™ are disc-shaped, microbiological reference materials. Each disc contains a quantified number of microorganisms (colony forming units; cfu), immobilized in a solid water soluble matrix.
Expiry date:	**JUL 2024**
Storage:	**-20 ± 5 °C;** store the mylar bag containing the plastic vials with the Vitroids™ unopened
Starting material:	**CECT 4300 batch 22-07-2015** (freeze-dried microorganism in a glass ampoule)
No. of passages:	**2** (upon receipt from cell culture collection CECT)

Sample: *Salmonella enterica* subsp. *enterica* serovar Enteritidis WDCM 00030 VT000303		
Certified value (geometric mean value)	Expanded uncertainty (log_{10} value)	Expected range
1.0E+02 cfu per disc	0.042	**4.6E+01 - 2.3E+02 cfu per disc**
Conditions:	**Trypcase soy agar / aerobic / 37 °C / 24 hrs**	
Date of testing:	11 AUG 2022	

cfu: colony forming units
The reference values are calculated by (US EPA Environmental Systems Monitoring Laboratory in Cincinnati) EMSL-CIN's computer program "BIWEIGHT". The measurement of uncertainty originates from the generated biweight standard deviation (SD) resulting from the biweight geometric mean value obtained during homogeneity testing. The expected range takes into account media batch to batch variability, which is done by multiplying the biweight standard deviation by 1.6.

Metrological traceability:	Details see "Certification process details" on page 2.
Measurement method:	The certified value is established by plate counting in accordance with ISO/IEC 17025[1].
Intended, correct use & handling instructions:	Please follow the instructions given in "General instructions for intended uses of this reference material" on page 3.
Health and safety information:	Please refer to the Safety Data Sheet (link on page 3) for detailed information about the nature of any hazard and appropriate precautions to be taken.
Accreditation:	Sigma-Aldrich Production GmbH is accredited by the Swiss Accreditation Service SAS as reference material producer under no. SRMS 0001 in accordance with international standard ISO 17034 [2].
Certificate issue date:	**07 SEP 2022**

ISO 17034
SRMS 0001

Dr. Thomas Buhrer – CRM Operations

Dr. Philipp Zell – Approving Officer

Sigma-Aldrich Production GmbH, Industriestrasse 25, 9471 Buchs, Switzerland; Tel +41-81-755-2511
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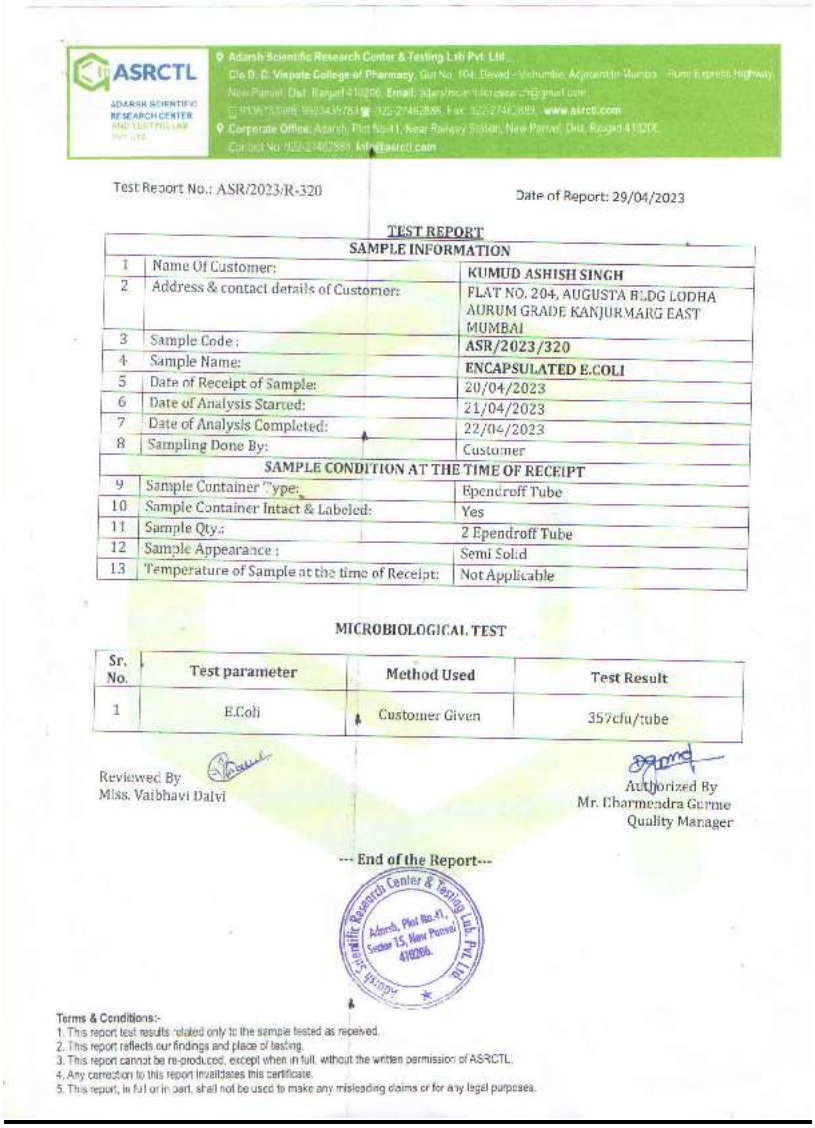
Appendix 7: Test report from testing laboratories



ASRCTL
ADARSH SCIENTIFIC RESEARCH CENTER AND TESTING LAB PVT. LTD.

Adarsh Scientific Research Center & Testing Lab Pvt. Ltd.
C/o D. D. Vispute College of Pharmacy, Gut No. 104, Devad - Vichumbe, Adjacent to Mumbai - Pune Express Highway, New Panvel, Dist. Raigad 410206. Email: adarshscientificresearch@gmail.com
[illegible] 9823439784 / 022-27462888, Fax: 022-27462889, www.asrctl.com
Corporate Office: Adarsh, Plot No.41, Near Railway Station, New Panvel, Dist. Raigad 410206.
Contact No 022-27462888, info@asrctl.com

Test Report No.: ASR/2023/R-320

Date of Report: 29/04/2023

TEST REPORT

SAMPLE INFORMATION		
1	Name Of Customer:	KUMUD ASHISH SINGH
2	Address & contact details of Customer:	FLAT NO. 204, AUGUSTA BLDG LODHA AURUM GRADE KANJURMARG EAST MUMBAI
3	Sample Code :	ASR/2023/320
4	Sample Name:	ENCAPSULATED E.COLI
5	Date of Receipt of Sample:	20/04/2023
6	Date of Analysis Started:	21/04/2023
7	Date of Analysis Completed:	22/04/2023
8	Sampling Done By:	Customer
SAMPLE CONDITION AT THE TIME OF RECEIPT		
9	Sample Container Type:	Ependroff Tube
10	Sample Container Intact & Labeled:	Yes
11	Sample Qty.:	2 Ependroff Tube
12	Sample Appearance :	Semi Solid
13	Temperature of Sample at the time of Receipt:	Not Applicable

MICROBIOLOGICAL TEST

Sr. No.	Test parameter	Method Used	Test Result
1	E.Coli	Customer Given	357cfu/tube

Reviewed By
Miss. Vaibhavi Dalvi

Authorized By
Mr. Dharmendra Germe
Quality Manager

--- End of the Report---

Adarsh Scientific Research Center & Testing Lab Pvt. Ltd. Adarsh, Plot No.41, Sector 15, New Panvel 410206.

Terms & Conditions:-
1. This report test results related only to the sample tested as received.
2. This report reflects our findings and place of testing.
3. This report cannot be re-produced, except when in full, without the written permission of ASRCTL.
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Adarsh Scientific Research Center & Testing Lab Pvt. Ltd.
C/o D. D. Vispute College of Pharmacy, Gut No. 104, Omale - Vadhumbe, Adjacent to Mumbai - Pune Express Highway, New Panvel Dist. Raigad 410206. Email: adarshscientificresearch@gmail.com
(M) 9136133088, 9923435763 (O) 022-27462886, Fax: 022-27462886, www.asrctl.com
Corporate Office: Adarsh, Plot No.41, Near Railway Station, New Panvel Dist. Raigad 410206.
Contact No. 022-27462886, info@asrctl.com

Test Report No.: ASR/2023/R-319 Date of Report: 29/04/2023

TEST REPORT

SAMPLE INFORMATION		
1	Name Of Customer:	KUMUD ASHISH SINGH
2	Address & contact details of Customer:	FLAT NO. 204, AUGUSTA BLDG LODHA AURUM GRADE KANJURMARG EAST MUMBAI
3	Sample Code	ASR/2023/319
4	Sample Name:	ENCAPSULATED SALMONELLA
5	Date of Receipt of Sample:	20/04/2023
6	Date of Analysis Started:	21/04/2023
7	Date of Analysis Completed:	22/04/2023
8	Sampling Done By:	Customer
SAMPLE CONDITION AT THE TIME OF RECEIPT		
9	Sample Container Type:	Ependrop Tube
10	Sample Container Intact & Labeled:	Yes
11	Sample Qty :	2 Ependroff Tube
12	Sample Appearance :	Semi Solid
13	Temperature of Sample at the time of Receipt:	Not Applicable

MICROBIOLOGICAL TEST

Sr. No.	Test parameter	Method Used	Test Result
1	Salmonella	Customer given	235cfu/tube

Reviewed By
Miss. Vaibhavi Dalvi

Authorized By
Mr. Dharmendra Gurme
Quality Manager

--- End of the Report---

Adarsh Scientific Research Center & Testing Lab Pvt. Ltd. Adarsh, Plot No.41, Sector 19, New Panvel 410206.

Terms & Conditions:-
1. This report test results related only to the sample tested as received.
2. This report reflects our findings and place of testing.
3. This report cannot be re-produced, except when in full, without the written permission of ASRCTL.
4. Any correction to this report invalidates this certificate.
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AUTOCAL SOLUTIONS PVT. LTD.: Plot No. PAP-R 149, 150 & 166, 167, Rabale MIDC,
(Behind Hotel Sai Prasad & Vista Inn), Pipeline Road, TTC Industrial Area, Navi Mumbai 400701
W: www.autocal.net, E: sonil@autocal.in, T: 8425998977

TEST REPORT

Report Issue Date : 20/04/2023

TEST REPORT NO.	ASPL/T2340242/OT-046				
Customer Name & Address	MS. KUMUD ASHISH SINGH Flat no 204, Augusta building, Lodha Aurum Grande, Kanjurmarg East, Mumbai Maharashtra 400042, India				
Order Reference	Test Request Form Dated: 20/04/2023				
DETAILS OF SAMPLE TESTED - As provided by customer					
Sample Name	Eppendorf Tubes Containing Encapsulated *Salmonella* & Encapsulated *E.coli* (Water Soluble Matrix 1.5 ml Semisolid)				
Batch No.	NA	Mfg. Date	NA	Best Before	NA
SAMPLING DETAILS					
Sample Drawn By	Picked By - Autocal Solutions Pvt. Ltd. On 20/04/2023		Sample Received on	20/04/2023	
Sampling Procedure	-				
Sample Container	Eppendorf Tubes		Sample Quantity	1.5ml X 4	
Analysis Start Date	22/04/2023		Analysis End Date	23/04/2023	

OBSERVATIONS AND RESULT

Discipline : Microbiological Group : Miscellaneous

PARAMETERS	RESULT	UNIT	METHOD
Enumeration of *E.coli*	330	cfu/tube	Customer Method
Enumeration of *Salmonella*	220	cfu/tube	Customer Method

Remark : Not Applicable

Note:

- This test report refers only to the sample tested.
- The testing results reported reflects the quality of sample at the time of testing.
- This test report should not be reproduced, except in full, without the prior permission of Autocal Solutions Pvt. Ltd.
- Any correction in this test report invalidates the test report.

Signature
Authorized Signatory
Designation

Vidya Kasar
Sr. Microbiologist

Page 1 of 1

..... END OF TEST REPORT .....

FRM/Q/023, 23/01/2023

H. O.: Unit No. 5, 10 & 11 R..by Ind. Estate PCS Ltd., Navghar, Vasai Road (E), Dist. Palghar 401 210.
W : www.autocal.net, E : sales@autocal.in, CIN No.: U72200MH2008PTC173116 T: 0250 - 2393502/03, 2392480

2300007476

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GEO-CHEM LABORATORIES PRIVATE LIMITED.
Pragati, Adjacent to Crompton Greaves
Kanjur Marg (E), Mumbai 400042
Tel :+91 22 61915100 Fax :+91 22 61915101
Email : laboratory@geochem.net.in

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&
Testing Company

GEO-CHEM LABORATORIES PVT LTD.-KANJURMARG

Quantitative Determination of E. coli & Salmonella

Geo Chem Lab Report Number: 2303K63715

Sample preparation and Inoculation

.Entire sample is dissolved/suspended in 10 ml of 0.1 % Peptone water and homogenized and allowed to stand for 30 minutes.

Serial dilutions are prepared as required. All dilution tubes are vortexed.

. 1 ml of solution is pipette out in each plate in duplicates. Add 20 ml of Soyabean Casein Digest Agar into each petridish.

Incubate at 37°C for 24 hours.

. Observations were taken after completion of incubation hours.

Observations are tabulated in Table 1 and 2

Observations

Table 1

DILUTION NO	NO. OF COLONIES PER PLATE	*TOTAL BACTERIAL COUNT Per gm/ml
10^{-1}	34	370 Cfu
10^{-2}	4	
10^{-3}	No Growth	
10^{-4}	No Growth	
10^{-5}	No Growth	
10^{-6}	No Growth	
10^{-7}	No Growth	
BLANK	No Growth	

Observations for Sample 1 E. coli (Eppendorf Tube)

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Tel.: +91 22 66383838 Fax: +91 22 66383800 Email: mumbai@geochem.net.in
CIN: U74220MH1964PTC013022 www.geochem.net.in

№ 569947

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GEO-CHEM LABORATORIES PRIVATE LIMITED.
Pragati, Adjacent to Crompton Greaves
Kanjur Marg (E), Mumbai 400042
Tel :+91 22 61915100 Fax :+91 22 61915101
Email : laboratory@geochem.net.in

GEO CHEM
International
Independent Inspection
&
Testing Company

Table 2

DILUTION NO	NO. OF COLONIES PER PLATE	*TOTAL BACTERIAL COUNT Per gm/ml
10^{-1}	31	310 Cfu
10^{-2}	3	
10^{-3}	No Growth	
10^{-4}	No Growth	
10^{-5}	No Growth	
10^{-6}	No Growth	
10^{-7}	No Growth	
BLANK	No Growth	

Observations for Sample 2 Salmonella (Eppendorf Tube)

For Geo-Chem Laboratories Pvt. Ltd.

Authorised Signatory

MANASI GOKHALE
Biological Analysis

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