# To optimize cell viability assay for probiotics

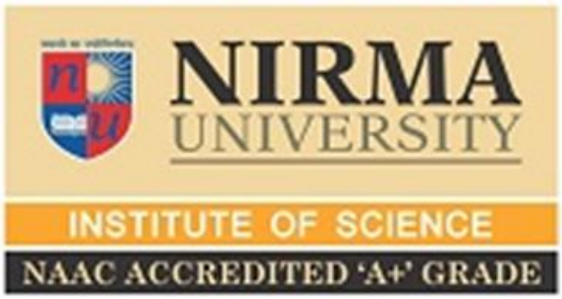
**A dissertation thesis submitted to Institute of Science**

**Nirma University in partial fulfilment of the requirement for degree of**

**MASTER OF SCIENCE**

**In**

**BIOTECHNOLOGY**

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NAAC ACCREDITED 'A' GRADE

# CERTIFICATE

This is to certify that the thesis entitled **"To optimize cell viability assay for probiotics"** submitted to the Institute of Science, Nirma University in partial fulfilment of the requirement for the award of the degree of M.Sc. in Microbiology, is a bonafide record of research work carried out by **Jhanvi Patel (21MBT050)** under the guidance of **Dr. Vijay Kothari**. No part of the thesis has been submitted for any other degree or diploma.

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# DECLARATION

I hereby kindly declare that the work entitled "**To optimize cell viability assay for probiotics**" is my original work. I have not copied from any other students' work or from any other sources except where due references or acknowledgement is made explicitly in the text, nor has any part been written for me by any other person.

**Date:**

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**Jhanvi Patel**

# INDEX

# Abbreviations

MTT - 3-(4,5-dimethylthiazol)-2,5-diphenyl-tetrazolium bromide

GRAS - General Recognised As Safe

CFU – Colony forming unit

DMF - Dimethylformamide

DMSO - Dimethyl sulfoxide

# 1. Introduction

The word "probiotic" which means "for life" is typically used to describe microorganisms that can benefit both people and animals (Grispoldi et al., 2020; Fuller., 1992). The organisms that are alive and present in the human intestine are known as probiotics. The phrase "live microorganisms that, when administered in suitable proportions impart a health benefit on the host" has now been added to the definition (Reid, 2016). The term was first used by Fuller in 1989 to describe "a live microbial feed additive that benefits the host animal by enhancing its gut microbial balance." (Fuller, 1992). They are an essential component of the human biological system and play a very important role in the healthy operation of our gastrointestinal tract (Okumura & Takeda, 2017). The dependence of the intestinal bacteria on the food makes it conceivable to adopt strategies to modify the flora in our bodies and to replace the harmful microbes by good microbes**.** It aids in preserving digestive health. It provides their hosts a variety of positive advantages. The three genera *Lactobacillus, Streptococcus* and *Bifidobacterium* are the most often used strains, however yeasts and *Enterococci* have also been employed as probiotics (Chow, 2002).

For microbial strains to be considered as probiotics, they must possess three qualities: (Mejía-Caballero et al., 2021)

(1) The ability to withstand the high acidity and bile salts of the digestive system.

(2) Exclusive competition and microbial activity

(3) Modulation of the immunological system of the host

Once probiotics have survived the host’s chemical barriers, they must face one of the most important barriers, the competition with a great diversity of microorganisms that form the gastrointestinal microbiota.

Probiotics are living microorganisms that are given to consumers in order to provide them with health benefits. By aiding with gut microbial balance, these bacteria contribute to maintaining health. *Bifidobacterium, Lactobacilli and Enterococci* are examples of the lactic acid bacteria that are the most distinctive active ingredients in probiotic supplements. Together, they strengthen the immune system of the host and are essential for protecting the organism from harmful bacteria. Probiotics are present in both dairy and non-dairy foods. They are frequently used as part of antibiotic therapy (for a variety of disorders), which destroys both the helpful and specifically targeted harmful bacteria existing in the microbial ecology of the digestive

tract. Obesity, diabetes, neurological disorders, even mood and behaviour have all been linked to the human microbiota (Soccol et al., 2019). Microbial inhabitants of humans, their ecology and their role in human health and disease are already hot research topics (Kothari et al., 2019).

Many probiotic formulations have already entered in the market and many more are expected in the near future. One or more microbial strains are present in these commercial probiotic formulations and some of them even make specific health benefits claims. As probiotics are living organisms, it is essential to count the number of viable microorganisms in the preparation properly and to inform the user of this information on the product label. Any probiotic product's potency is directly correlated with the quantity of bacteria it contains per unit volume or weight. The minimum number of spores or CFU contained in each individual pack of the probiotic product is mentioned in many of the goods (Kothari et al., 2019). Nevertheless, this number might alter when the product is transported and stored and an appropriate temperature may not always be kept exactly as it should be. Because of these include living organisms or spores that might germinate, these cells or spores may become inactive for a number of reasons.

A microbial species may be identified by using a variety of techniques, but not all of them are equally effective for all classes of bacteria. The majority of probiotic products contain bacterial species and the majority of them contain strains from the genera *Bifidobacterium* and *Lactobacillus* (Kothari et al., 2019).

Each commercial probiotic product should be properly characterized on the following criteria: (Kothari et al., 2019)

A. Identification of the process organism's genus, species, and strain

B. Microbial load (per mL or g) of the product during manufacture and at the end of its shelf life

C. Biological effect

**1.1 Bacteria as a probiotics:**

Lactobacillus species are a group of bacteria characterized by their rod-shaped structure, gram-positive nature and facultative ability, meaning they can adapt to different oxygen conditions. They do not form spores. One of their main end products is the fermentation of carbohydrates into lactic acid (Goldstein et al., 2019). Due to their complex taxonomy, these bacteria frequently require molecular speciation in order to be identified. They are abundant in nature and have a variety of industrial applications, particularly in the fermentation of cheese and other dairy products (Aguirre et al., 1993; Danilenko et al., 2021). Lactobacilli are commensal bacteria that are present in significant numbers in both the female genitourinary tract and the human gastrointestinal tract. They are an essential component of the gut and vaginal microbiota and are helpful in preventing chronic disorders like inflammatory bowel disease (Troche et al., 2020) .

- **Reasons why bacteria is a probiotic:**

The following criteria were used to classify the bacterium as a probiotic based on the three facts: (Krishnakumar, 2019)

1. The bacteria must be safe to consume, according to the law of Generally Recognized As Safe (GRAS) , which means only safe bacteria are allowed to be receive on the market.

2. Scientific proof must be for the specific bacterium, and it must provide measurable health benefits.

3. The bacteria must be alive when they enter the human digestive tract. It must be suggested with an adequate dose from the time of preparation to the time of consumption.

The two bacterial genera that are most often used in probiotic products for human consumption are *Lactobacillus* and *Bifidobacterium* (Sadeghi-Aliabadi et al., 2014; Salminen et al; 1998). Probiotic preparations must adhere to high standards for functionality, quality and safety (Vankerckhoven et al., 2008). The fact that they contain the precise amount of live cells stated on the product label is a crucial quality requirement. Some researchers have discovered that the cell counts of *Lactobacilli* in commercial products are considerably lower than what is reported or claimed by the manufacturers. This suggests that the actual number of *Lactobacilli*

in these products may be significantly less than what consumers are led to believe. (Lin et al., 2006).

To confirm the claim, here we have used Sporlac powder [Sanzyme (P) Ltd.] containing lyophilized spores of *Lactobacillus sporogenes*. Each sachet is of 1 gram containing 15 million spores as per the claim.

**1.2 Yeast as a probiotics:**

Yeasts are unicellular, eukaryotic microorganisms that are a part of the kingdom Fungi, of which *Saccharomyces* is the most common genus. Among them, *Saccharomyces boulardii* is a widely researched species that is utilized in many nations as a therapeutic and preventive agent due to its probiotic properties, including its resistance to the acidic environment of the gastrointestinal tract and improvements in the gut microbiota (Kelesidis and Pothoulakis, 2012; Hadjimbei et al., 2020). It is a mesophilic, non-pathogenic yeast that differs from *S. cerevisiae* in terms of metabolism and physiology due to, among other things, its tolerance to bile acids, resilience to low pH, and optimal development temperature of 37ºC (McFarland, 2017; Souza et al., 2021).

*Saccharomyces boulardii*, a yeast probiotic that has been patented, has been proven to be effective through double-blind studies. It is a patented yeast preparation and has exhibited positive outcomes in research. *Saccharomyces boulardii* a yeast, is widely used in many countries as a preventive and therapeutic treatment for diarrhea and other gastrointestinal disorders caused by the use of antimicrobial agents (Czerucka et al., 2007).

*S. boulardii* has several additional attributes that provide probiotic properties, such as the beneficial effects against enteric pathogens, including the production of compounds that neutralize microbial toxins, prevention of bacterial adherence and translocation in intestinal epithelial cells and modulation of the host cell signaling pathway associated with the pro-inflammatory response in bacterial infection (Moslehi-Jenabian et al., 2010) **.**

It has numerous characteristics that make it a promising probiotic agent, including the ability to survive passage through the GI tract, a preferred temperature range of 37ºC and the ability to suppress the development of many microbial pathogens both in vitro and in vivo. Nevertheless, *S.boulardii* differs from prokaryotic bacteria in that it is a member of the class of simple eukaryotic cells, which also includes fungus and algae (Czerucka et al., 2007). Econorm® by Dr.Reddy's is taken as a market source for lyophilized cells of *S. boulardii*.

### 1.3 MTT Assay:

Cell viability and proliferation are frequently evaluated using the MTT test, which is based on the enzymatic reduction of the water-soluble, yellowish tetrazolium salt 3-(4,5-dimethylthiazol)-2,5-diphenyl-tetrazolium bromide (MTT) to purple formazan. The full solubilization of cells, formazan and the stability of the colored solution are required for the MTT test to work accurately. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO), buffered generated the greatest results, according to a comparison of several solubilization solutions (Benov L., 2021).

## 2. Literature review

Viable probiotic strains are offered as freeze-dried supplements, medicinal preparations, or incorporation into fermented food products commercially (Holzapfel and Schillinger, 2002). According to FAO/WHO standards, a probiotic must have a concentration of $10^6$ to $10^7$ CFU (Colony Forming Units) of live probiotic bacteria in order to make health benefit claims.

Probiotic products must contain enough bacteria to last until the expiration date(Fasoli et al., 2003). In order to help the host's health, probiotics must thus include particular strains and retain a specified percentage of living cells (Mattila-Sandholm et al., 2002).

For probiotic cultures to be used in functional meals, retention of the probiotic bacteria's viability provides a significant marketing and technological barrier (Desmond et al., 2002; Mattila-Sandholm et al., 2002). Several active cultures die during the production, storage, or transportation of the completed product as well as throughout the passage to the gut. As a result, the majority of cells die even before experiencing any health advantages (Siuta-Cruce and Goulet, 2001; Park et al., 2002). With dried cultures, there has been a severe issue with shelf stability.

Market research found substantially lower numbers in the items, even before their expiry date (Talwalkar et al., 2001). Since probiotics shelf lives are unpredictable, the industry had difficulty defending label claims (Siuta-Cruce and Goulet., 2001). In an effort to compensate for cells that die during storage, excesses of 50 to 200% cells have been added to products (Thantsha et al., 2012).

Industrial-scale production of probiotics and prebiotics faces a number of challenges, including: (Figueroa-Gonzalez et al., 2011)

(i) The use of cutting-edge methods and cost-effective sources for prebiotic production
(ii) The production of probiotics at a low cost
(iii) The enhancement of probiotic viability following storage, during the production of the functional food, and during transit through the stomach.

Such commercial probiotic products on the market might raise a number of problems for regulatory agencies and informed consumers, including: (Kothari et al., 2019)

- Whether the product has the same species and/or strain of bacteria, as described on its pack?
- Whether the product contains the number of bacteria listed on the product label?
- Can the bacteria multiply inside the product container during storage, increasing their quantity during consumption?
- Does the number of bacteria listed on the product label remain the same from manufacture through storage to consumption?

**2.1 WHO Recommendation:**

Probiotic bacteria should be alive from the time they are processed, packaged, and shipped by the manufacturer until they are ingested and have a chance to settle in the gut. As a result, there are two major obstacles to probiotic delivery: shelf-stability and stomach (gastric acid) environment passage.

**2.2 Minimum viable cells:**

Different nations have established minimum viable cell counts that must be included in a probiotic product for it to be effective. For instance, Australia requires $10^6$ live organisms per gram as a minimum.

Delivering a viable colony of live bacteria to the target area of the intestines, where they are thought to be active, is the key problem now facing the probiotics business globally.

Maintaining the probiotic bacteria's viability is a significant obstacle as they tend to expire during production, preservation, or while in transit to the final product. Moreover, a considerable amount of them get eradicated while passing through the stomach to reach the intestines.

### 2.3 Probiotics shelf-life:

The industry has raised worry about probiotics viability at room temperature. Before they reach the ultimate customer, most probiotics products must spend 2 to 18 months on the drugstore shelf. During this time, the live bacteria gradually deteriorate and disappear as a result of oxidation and other storage-related stress.

The "enemies of stability" have been referred to as oxygen, water (moisture), and high temperatures. When probiotic bacteria are subjected to extreme temperatures, excessive wetness, and air exposure, their stability declines, which means they start to die. Due to the detrimental effects of low temperature and crystal formation on bacterial organisms, refrigerated items also have limited shelf life.

The majority of probiotic bacteria degrade and lose viability as they age. During storage, the amount of live bacteria steadily declines over time. Market research has found that the numbers in the items before their expiration dates are substantially lower. Because of this, shelf life is uncertain, and most probiotics struggle to live up to label claims (Valobiotics, n.d).

## 3. Materials & methods

Minimal media (HiMedia G013-500G)

MTT reagent (3-(4,5 Dimethyl thiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) (HiMedia TC191-1G)

Normal saline (0.85% W/V NaCl in distilled water) (Merk Life Science MH1M711297)

DMSO (dimethyl sulfoxide) (Merk Life Science SE6S660292)

*Lactobacillus sporogenes* spores (Sanzyme) (Batch No – MBA22038)

*Saccharomyces boulardii* cells (Dr.Reddy's) (Batch No – 6831)

- ## Methods:

- **Standardized MTT Protocol for *Lactobacillus sporogenes*:**

The MTT assay to measure the metabolic activity of *Lactobacillus sporogenes* in response to different amounts of spores. Here given is the detailed method for the same:

- Sterilized Minimal media (HiMedia G013-500G) of volume 9 mL each were taken in U-shaped Centrifuge tubes (50 mL).
- Different amounts (0.5 to 4 g**)** of *Lactobacillus spores* were added to each tube to get the different concentrations of *Lactobacillus sporogenes*.
- Vegetative cells of *Lactobacillus plantarum* was used a positive control to check the efficiency of the standardized protocol along with an abiotic control (minimal media + 1 mL N -saline).
- The mixture was vortexed to ensure a homogeneous solution is made.
- 1 mL of 0.3% MTT(in distilled water) was added to each tube in a dark environment and the tubes were then incubated at 35ºC in the dark for 18 hours ± 20 min.
- After incubation the tubes were, centrifuged at 7500 rpm for 15 minutes at 25ºC and the supernatant was discarded.
- 10 mL of DMSO was added ensuring each pellet is mixed well by shaking to solubilize the formazan product from the bacteria.
- The contents of the tube were vortexed and centrifuged again at 7500 rpm for 15 minutes at 25ºC.
- The absorbance of the purple supernatant measured at 540 nm using a spectrophotometer to indicate the metabolic activity of the bacteria.

- **Standardized MTT protocol for *Saccharomyces boulardii*:**

The MTT assay to measure the metabolic activity of *S. boulardii* in response to different amounts of lyophilised cells. Here given is the detailed method for the same:

- Sterilized Minimal media (HiMedia G013-500G) of volume 9 mL each were taken in V-shaped microfuge tubes (15 mL).
- Different amounts of (1 to 60 mg) *Saccharomyces boulardii* were added to each tube.
- Vegetative cells of *S. cerevisiae* were used a positive control to check the efficiency of the standardized protocol along with an abiotic control (minimal media + 1 mL N-saline).
- The mixture was vortexed to ensure a homogeneous solution is made.
- 1 mL of 0.3% MTT (in distilled water) was added to each tube in a dark environment and the tubes were then incubated at 28ºC in the dark for 3 hours ± 20 min.
- After incubation the tubes were, centrifuged at 7500 rpm for 15 minutes at 25ºC and the supernatant was discarded.
- 10 mL DMSO was added ensuring each pellet is mixed well by shaking to solubilize the formazan product from the bacteria.
- The contents of the tube were vortexed and centrifuged again at 7500 rpm for 15 minutes at 25ºC.
- The absorbance of the purple supernatant measured at 540 nm using a spectrophotometer to indicate the metabolic activity of the bacteria.

## 4. Results

Several incubation durations including 6, 18, and 24 hours were used for MTT tests. At 6 hours, we received lower results as compared at 18 and 24 hours. We proceeded with 18 hours since the results at 18 and 24 hours were similar.

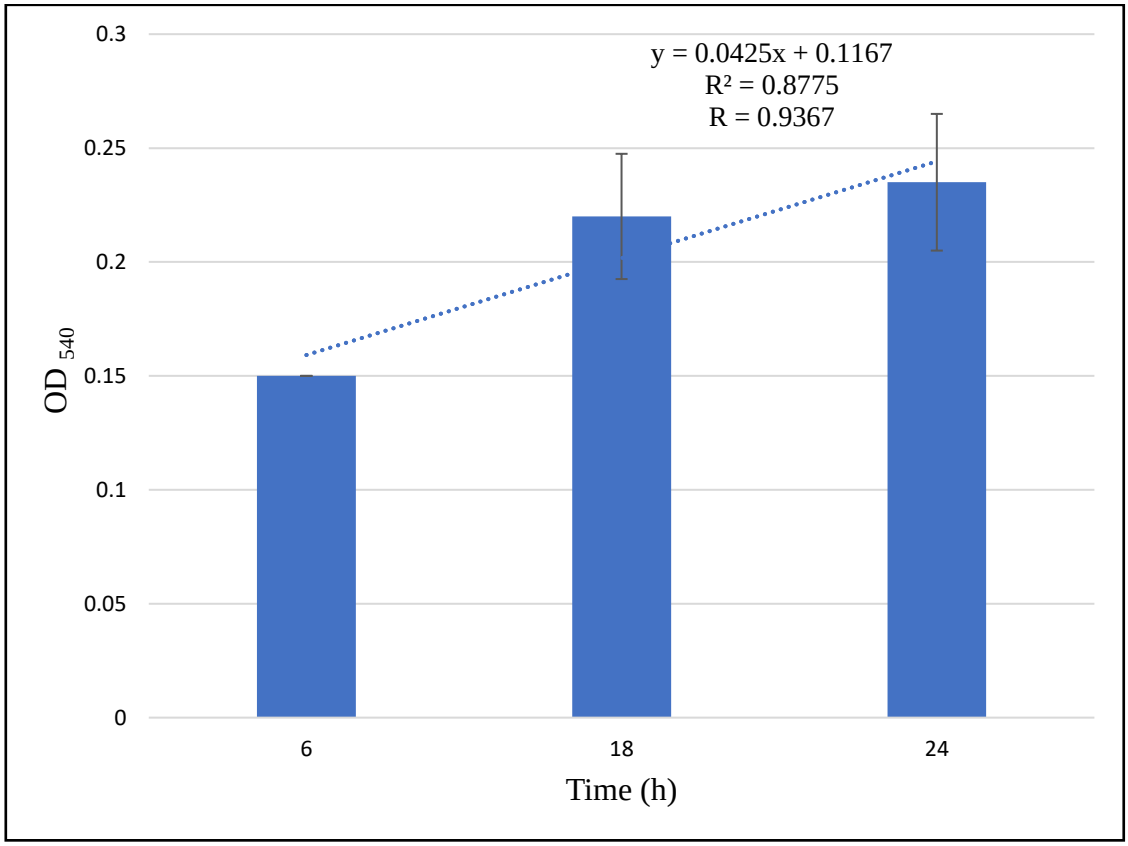


**Fig 1: Optimization of incubation time period with MTT for *Lactobacillus sporogenes***

The graph shows the spectrophotometric readings at 540 nm of 1 gm of *Lactobacillus sporogenes* spores in minimal media following incubation with MTT reagent for 6, 18, and 24 hours at 35°C. It demonstrates that the OD value at 18 hours was higher than that at 6 hours, while it remained similar with that at 24 hours.

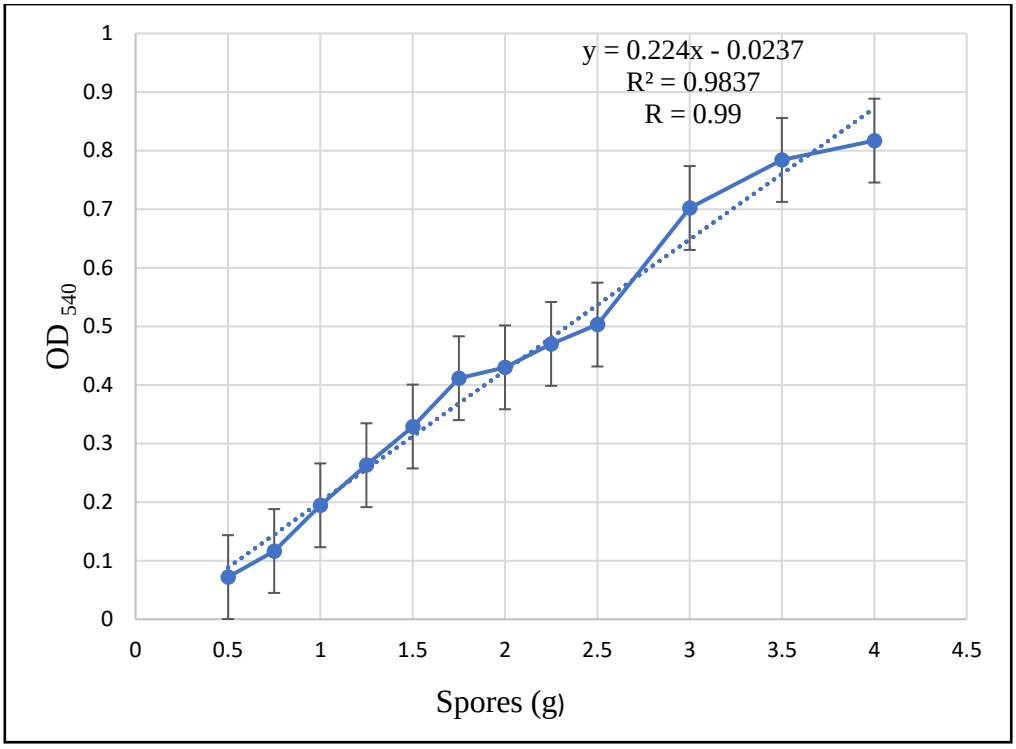


**Fig 2(A): MTT assay of *Lactobacillus sporogenes* spores for cell viability for amount (g) vs OD at 540 nm.** The graph shows mean values of 3 independent experiments of various amount of *Lactobacillus sporogenes* spores against its absorbance at 540 nm. Vegetative cells of *Lactobacillus plantarum* as positive and Abiotic control (N-saline**)** added in minimal media.

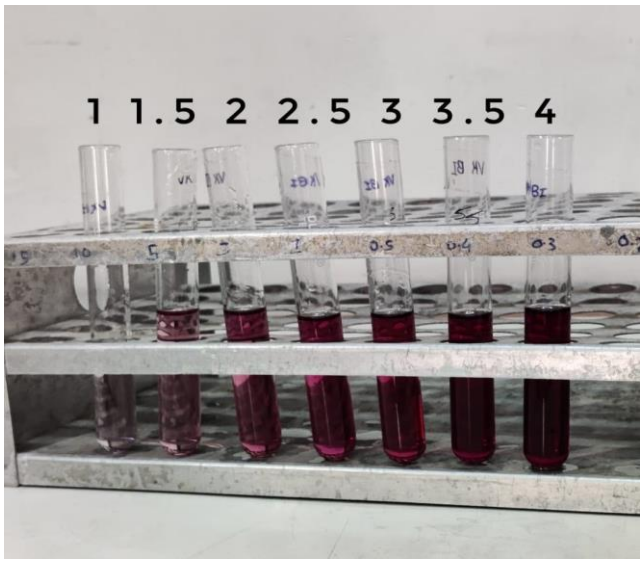


**Fig 2(A) : *Lactobacillus sporogenes* spores performed in test tubes by MTT assay.** During the MTT assay of *Lactobacillus sporogenes* spores performed in test tubes, the addition of DMSO to the different amounts of MTT-stained spores resulted in the formation of a purple color.

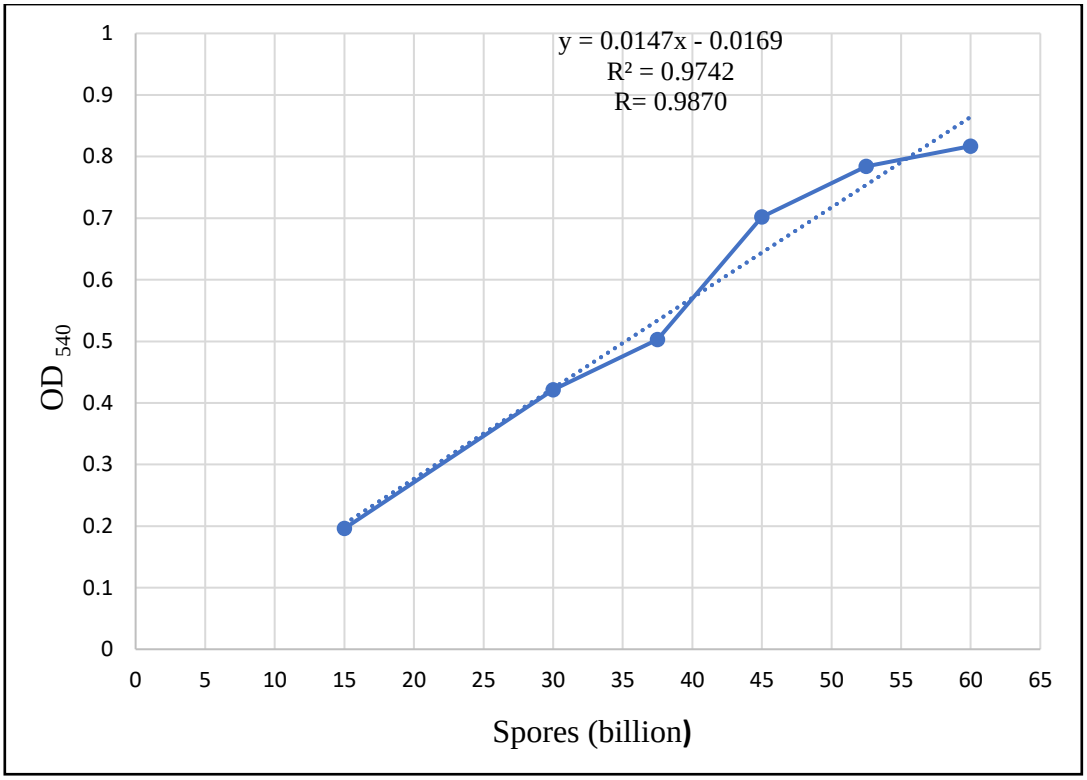


**Fig 3: The graph displays the relationship between the number of spores (billion) of *Lactobacillus sporogenes* spores vs the OD at 540 nm obtained from the MTT assay.** The data shows the number of spores and the spectrophotometric OD measured, indicating that as the number of spores increases, the absorbance at 540 nm also increases.

**Table 1: The table shows number of spores (billion) of *Lactobacillus sporogenes* spores in g vs the OD at 540 nm**

| Quantity(g) | Spores (billion) | OD at 540 nm |
|---|---|---|
| 1 | 15 | 0.196 |
| 1.5 | 22.5 | 0.329 |
| 2 | 30 | 0.4215 |
| 2.5 | 37.5 | 0.503 |
| 3 | 45 | 0.702 |
| 3.5 | 52.5 | 0.784 |
| 4 | 60 | 0.817 |

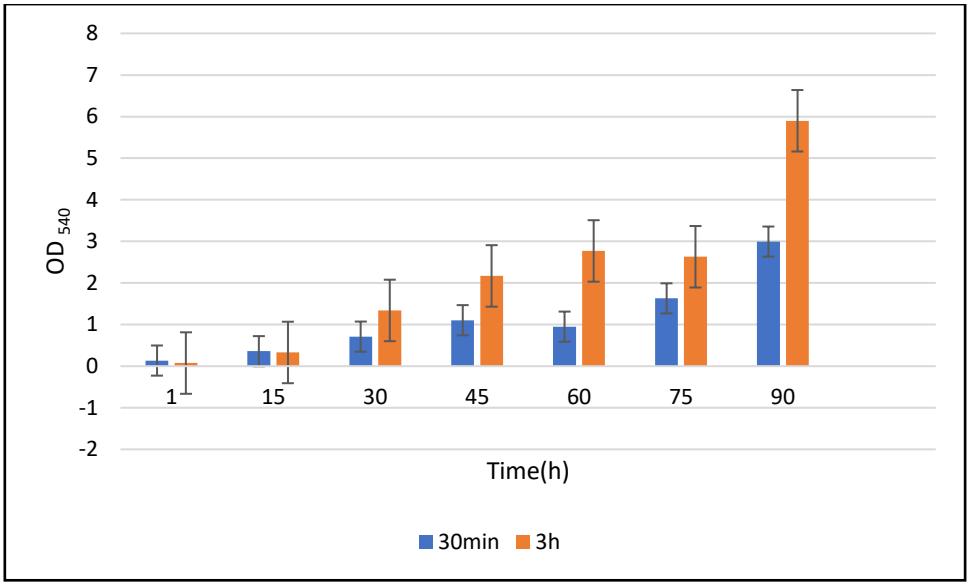


**Fig 4: Optimization of incubation time period with MTT for *S. baulardii*.** The graph shows the spectrophotometric readings at 540 nm of 1 mg of *S. baulardii* lyophilized cells in minimal media following incubation with MTT reagent for 30 min and 3hours at 35°C. It demonstrates that the OD value at 3 hours was higher than that at 30 min.

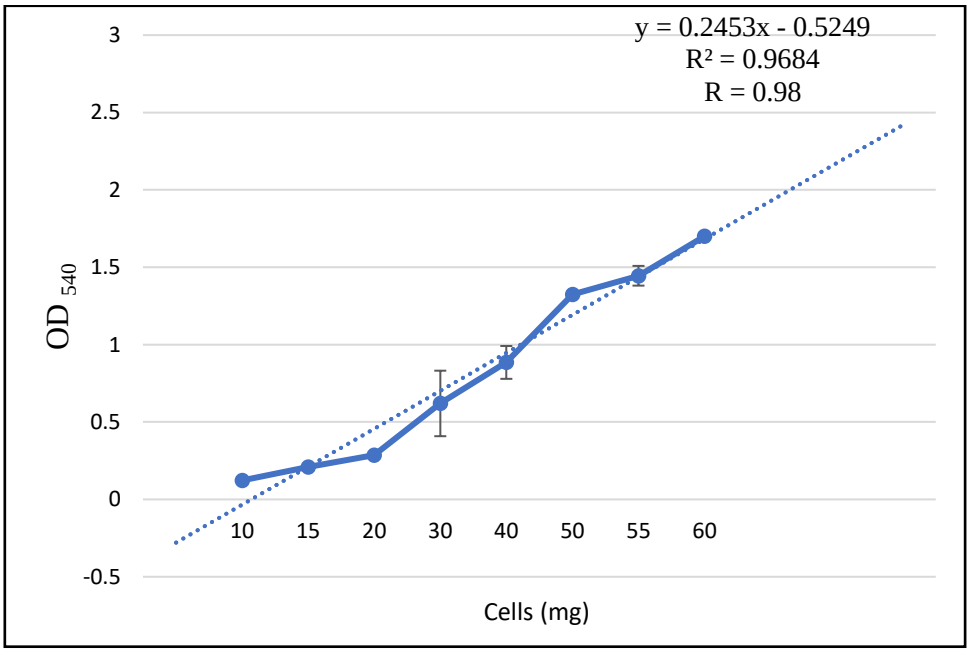


**Fig 5: MTT assay of *S. baulardii* *for* the amount of cells (mg) vs OD at 540nm.**The graph illustrates the absorbance at 540 nm of varying numbers of cells from three separate experiments using mean values. The trendline of the graph shows when amount of cells increases OD also increases.

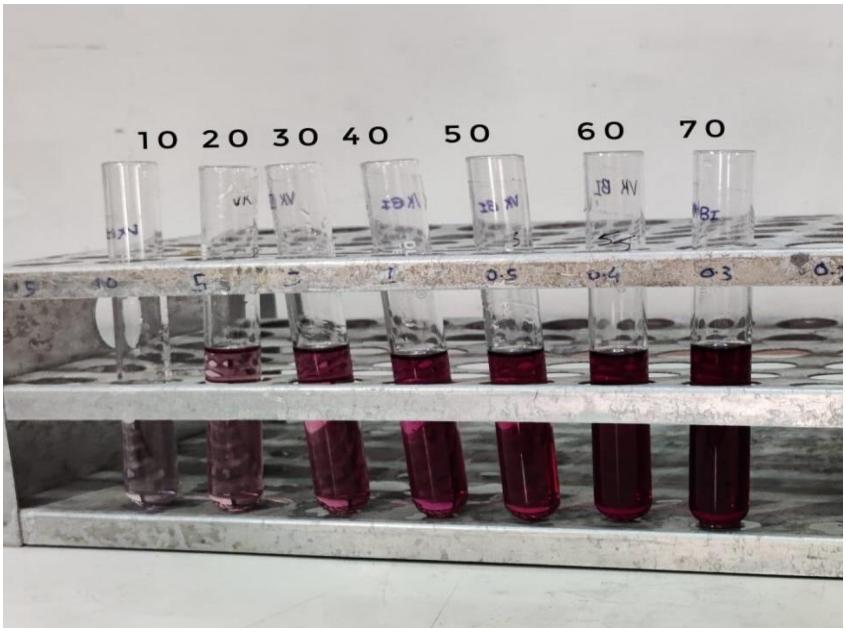


**Fig 5(A): Lyophilized *Saccharomyces boulardii* cells performed in test tubes by MTT assay.** During the MTT assay of Lyophilized *Saccharomyces boulardii* cells performed in test tubes, the addition of DMSO to the different amounts of MTT-stained spores resulted in the formation of a purple color.

## 5. Discussion

To ensure accurate assessment of cell viability, the MTT assay protocol must be standardized for each probiotic strain. This can involve adjusting parameters such as container surface area and incubation time with MTT. In this study, modifications were made to these parameters, and a range of detectable cell amounts (weight) that produced significant absorbance in the spectrophotometer was identified. Also, serial dilution (in minimal media) up to $10^4$ dilutions was performed twice followed by spreading in MRS agar. The colonies were less than 30 indicating no significance of CFU method for the quantification of probiotics. For *Lactobacillus. sporogenes* spores, a minimum detectable amount of 0.5 g was found, and the maximum weight that could be tested varied with container size, up to 4 g. Similarly, for lyophilized *S. boulardii* cells, a detectable range between 10 mg and 60 mg was observed, with higher amounts requiring more MTT reagent. Whereas, *Lactobacillus sporogenes* less than 0.5 g were not detectable because MTT did not came in contact with particular amount of spores and for *S. baulardii* less than 10 mg were not detectable because amount was less and above 60 mg there were no change in OD hypothesizing that more volume of MTT might be

required. The study also found that the size of the vessel used for the assay can impact on detectable range.

Initially, experiments were conducted in 15 mL Falcon tubes, which were found small for the spores. However, after changing the vessel size from 15 mL to 50 mL the amount of spore was detected for reliable results. Therefore, to use the MTT assay on an industrial scale, it must be standardized based on the microorganisms and their growth forms (spore or vegetative).

## 6. Conclusion

The MTT assay is an important for measuring cell viability and proliferation, but it is crucial to standardize the assay parameters each time it is used. This includes accounting for the surface area and volume of the container, which can vary depending on the number of cells or spores being tested. The incubation time for the reagent should also be carefully considered, as this can vary based on the type of microorganism used and whether it is in the vegetative or spore form. Additionally, the volume of MTT reagent used must be optimized to ensure that the range of linearity is appropriate for the number of cells being tested. By taking these factors into account, researchers can ensure that their MTT assay results are accurate, reliable, and consistent across experiments.

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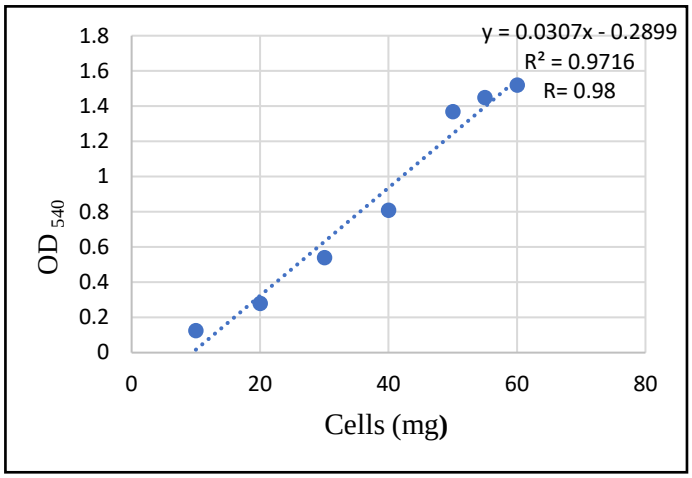
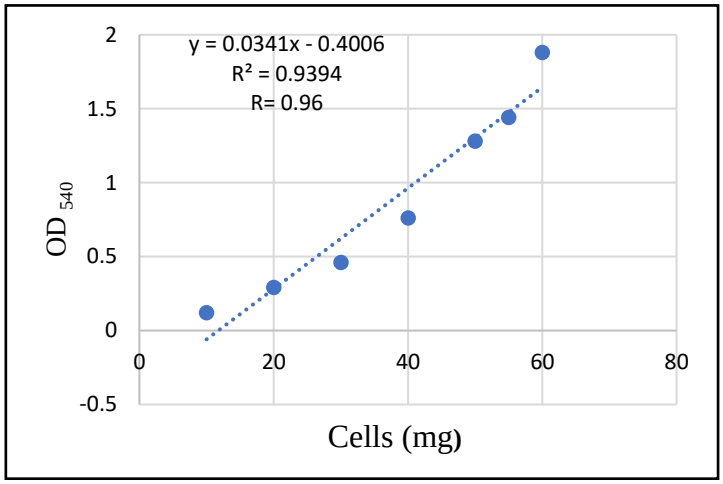
# 8. Appendix

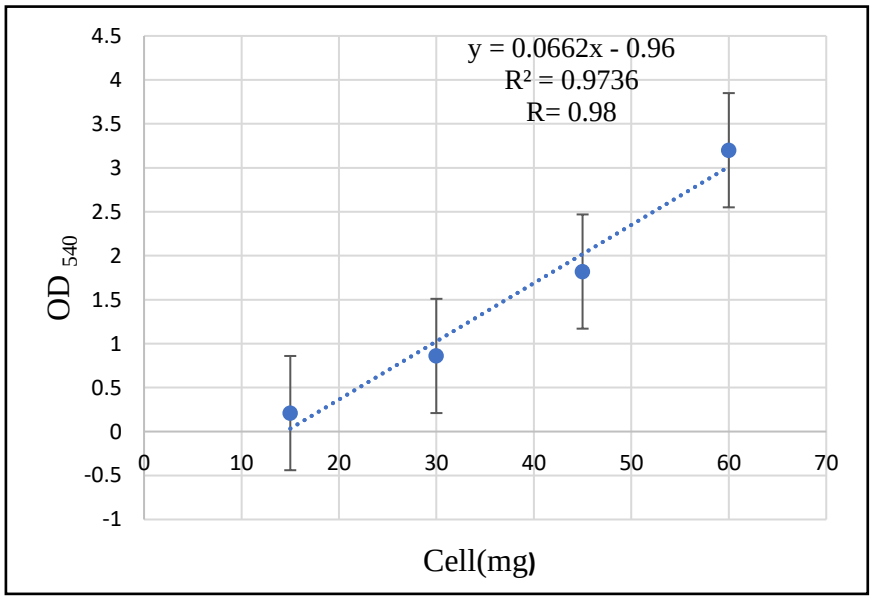
- **For *S. baulardii* :**




**(A)**



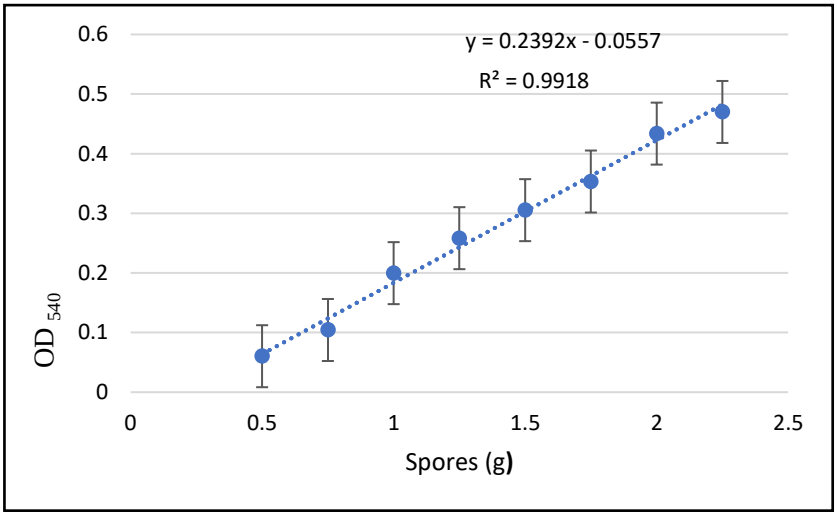

**(B)**



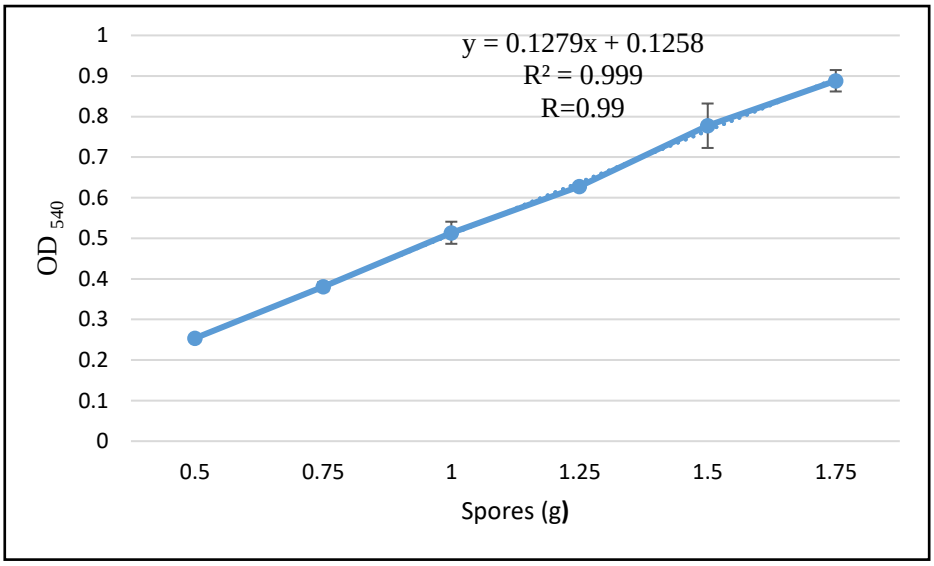

**(C)**

**Fig 6 : (A, B & C )** All the graphs mentioned here are of individual experiments performed and standard deviation is shown. It indicates the OD at 540 nm vs amount of cells in mg for *S. baulardii*.

- **For *L. sporogenes* :**




**(A)**




**(B)**

**Fig 7 (A & B) :** All the graphs mentioned here are of individual experiments performed and standard deviation is shown. It indicates the OD at 540 nm vs amount of cells in mg for *Lactobacillus sporogens.*