

## STUDIES ON ACTINOMYCETE ISOLATES FOR PROBIOTIC ACTIVITY

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### ABSTRACT

The probiotic concept is having lots of different applications in human and animal health. Probiotics are the living organisms which, when administered in adequate amounts confer a health benefit on host. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms. The beneficial effects produced by probiotics are like lactose intolerance, immune system, traveller's disease, cancer, dysbiosis, cholesterol reduction, etc. The present research work is to study existing actinomycetes isolates for probiotic activity and have the surviving capability at low pH and bile tolerance towards bile salts. Among the

existing isolates of VMS 20-30 isolates, VMS-30 was selected based on their antimicrobial activity tested against *Staphylococcus aureus* and *Escherichia coli*. Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below. As the existing actinomycete isolate was tested for their ability to tolerate the low pH, it was observed that there was no change in the growth of the inoculated organism. According to the results VMS-30 strains were resistant to 0.2%, 0.3%, 0.4%, 0.5% bile salt. The isolate was able to grow in bile salt as they survive.

**KEYWORDS:** Probiotics, Bile tolerance, Acid tolerance, Actinomycetes.

## INTRODUCTION

The word 'probiotic' comes from Greek language 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The term 'probiotic' firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. The meaning was improved to the closest by Parker in 1974. Parker defined 'probiotic' as 'substances and organisms which contribute to intestinal microbial balance'. In 1989, the meaning use today was improved by Fuller as, 'Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition, 'A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract, 'A live microbial food ingredient that is beneficial to health, 'A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host, 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' is accepted by FAO/WHO (report in October 2011). (Sanders *et al.*, 2003) The probiotics which are used to feed both man and animals are shown in the Table 1.1.

**Table 1.1: Microorganisms applied in probiotic products.**

| <i>Lactobacillus</i> species | <i>Bifidobacterium</i> species | Others  |
|------------------------------|--------------------------------|---|
| <i>L. acidophilus</i>        | <i>B. bifidum</i>              | <i>Enterococcus faecalis</i>                                  |
| <i>L. rhamnosus</i>          | <i>B. breve</i>                | <i>Enterococcus faecium</i>                                   |
| <i>L. gasseri</i>            | <i>B. infantis</i>             | <i>Pediococcus acidilactici</i>                               |
| <i>L. casei</i>              | <i>B. longum</i>               | <i>Saccharomyces boulardii</i>                                |
| <i>L. crispatus</i>          | <i>B. lactis</i>               | <i>Leuconostoc mesenteroides</i>                              |
| <i>L. plantarum</i>          | <i>B. adolascensis</i>         | <i>Streptococcus salivarius</i> subsp.<br><i>Thermophilus</i> |
| <i>L. johnsonii</i>          |                                | <i>Lactococcus lactis</i> subsp. <i>Lactis</i>                |

### 1.2. Effects of Probiotics on Health

Effects of probiotics on health are managing lactose intolerance, Improving immune system, Prevention of colon cancer, Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence), Lowering blood pressure, Reducing inflammation, Reduction of allergic symptoms, Beneficial effects on mineral metabolism, particularly bone density and stability, Reduction of *Helicobacter pylori* infection, Suppression of pathogenic microorganisms (antimicrobial effect), Prevention of osteoporosis, Prevention of urogenital infections.

### 1.3. Mechanism of Probiotics

Probiotic microorganisms are considered to support the host health. There are studies on how probiotics work. So, many mechanisms are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms (Salminen *et al.*, 1999) are Production of inhibitory substances, Blocking of adhesion sites, Competition for nutrients, Stimulating of immunity, Degradation of toxin receptor.

### 1.4. Selection Criteria for Probiotics

The selection criteria can be categorized in four basic groups Appropriateness, technological suitability, competitiveness, performance and functionality. Strains which have these criteria should be used in order to get effective on health and functional probiotic strains. Probiotics are chosen by using the criteria in (Çakır 2003).

#### Acid and Bile Tolerance

Bacteria used as probiotic strains are joined in the food system with a journey to the lower intestinal tract via the mouth. probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity, high acidic conditions in the stomach and enter the upper intestinal tract which contain bile. In this stage strains should have the ability to resist the digestion processes. It is reported that time at the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile.

#### Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Some milk products were used to isolate potential probiotic bacteria and determination of their possible antimicrobial activities.

#### Safety Aspects of Probiotics

Safety aspects of probiotic bacteria includes ,Strains for human use are preferred to be human origin and are isolated from healthy human gastrointestinal tract, have to be non-pathogenic and do not deconjugate bile salts, they have to no history of relationship with diseases like, infective endocarditis or gastrointestinal tract disorders.

## MATERIALS AND METHODS

**Chemicals:** All the chemicals and media used in this study were from Hi-Media chemicals. All the glassware used in the experiment were manufactured by Borosil.

### Media used

- For screening actinomycetes as probiotics Starch Casein Agar medium was used.
- For evaluation of marketed probiotic capsules MRS (mann rogosa sharpe) agar medium was used.
- Nutrient agar medium and Sabouraud medium are used for cultivation of bacterial cultures.

### 3.2. Isolates Tested For Probiotic Activity

In the present study existing cultures of actinomycete isolates were tested for probiotic activity. Among the existing VMS 20-30 isolates, VMS-30 isolate was selected because of its good antibacterial activity.

### 3.3. Properties of Probiotics

#### 3.3.1. Determination of acid tolerance (Kirtzaliduo *et al.*, 2011)

Resistance to pH 3 is often used in vitro assays to determine the resistance to stomach pH. VMS-30 isolates were freshly sub-cultured and starch casein broth was prepared and adjusted to pH 3 using hydrochloric acid (6.0 N). The starch casein broth was initially inoculated  $10^6$  CFU/ml. Samples were incubated and Cells were serially diluted 10-fold in phosphate buffer (0.1 M pH 6.2) in order to neutralize the medium acidity. The residual and viable count was determined by dilution and plate counting techniques after incubation. The survival rate was calculated as the colonies grown to the initial culture concentration.

#### 3.3.2. Determination of bile salt tolerance (Kirtzaliduo *et al.*, 2011)

The mean intestinal bile concentration is believed to be 0.3% (w/v). Determination of bile salt tolerance was performed by pour plate technique. Starch casein agar media was supplemented with 0.2%, 0.3%, 0.4%, 0.5% bile salts and inoculated with  $10^6$  CFU/ml from freshly sub-cultured VMS-30 strains and poured on to the petriplates and incubated for 4-7 days. And control plate was inoculated with VMS-30 cell culture was incubated without bile salt.

### 3.3.3. Antimicrobial activity (Williams *et al.*, 1983)

For antimicrobial activity agar overlay method was used. Active cultures were spotted on to the surface of starch casein agar media. The starch casein agar plates were incubated for growth. The next step is to prepare indicator microorganisms. Overnight indicator cultures were inoculated into soft agar containing 0.7% agar and this inoculated agar was overlaid on to the starch casein agar media. These plates were incubated according to the appropriate conditions for indicator microorganisms. At the end of the incubation, inhibition zone diameters (surrounding the spotted isolates) were measured.

## 3.4. Evaluation of Probiotic Capsule

### 3.4.1. Specifications of probiotic capsule

**Table 3.4: Specifications of probiotic capsule.**

| Capsule of probiotics LOBUN™         |  |
|--------------------------------------|--|
| Each hard gelatine capsule contains: | <b>Probiotics:</b> Not less than 15 billion cells of a blend of <i>Streptococcus thermophilus</i> , <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium longum</i> , <i>Bacillus coagulens</i> .<br>Appropriate overages added to compensate loss on storage<br>Empty hard gelatine capsule contain approved colours |
| Dosage                               | 3 capsules daily or As directed by physician   |
| Storage                              | Store in cool place, protected from moisture   |
| Mfg.Lic.No.                          | 41/UA/LL/SC/P-2011   |
| TM                                   | Trade mark under registration  |
| Manufactured and marketed by         | Sanzyme(P) Ltd.  |
| B.NO.                                | DLC-16003  |
| MFG.                                 | March 2016   |
| EXP.                                 | August 2017  |

Each capsule contain  $1.5 \times 10^8$  cells. For evaluation of probiotic capsules the cells should be serially diluted until the dilution contains 10 to 100 cells per 10ml. Capsule cells are diluted using sterile saline.

**Preparation of sterile saline:** 0.9% w/v sodium chloride solution is generally considered as saline solution. 0.9g of sodium chloride is added to 100ml water.

**Preparation of capsule cells suspension:** Initially one capsule was suspended in to 10ml sterile saline solution which contains  $1.5 \times 10^8$  cells and from the above 10ml inoculated sterile

saline solution was serially diluted to  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$ . Where last two dilutions contains 100 and 10 cells per 10ml respectively.

### 3.4.2. Determination of acid tolerance (Sahadeva *et al.*, 2011)

Resistance to pH 3 is often used in vitro assays to determine the resistance to stomach pH. Marketed probiotic samples were freshly sub-cultured and MRS broth was prepared and adjusted to pH 3 using hydrochloric acid (6.0 N). The MRS broth was initially inoculated with  $10^6$  CFU/ml. Samples were incubated and Cells were serially diluted 10-fold in phosphate buffer (0.1 M pH 6.2) in order to neutralize the medium acidity. The residual and viable count was determined by dilution and plate counting techniques after incubation. The survival rate was calculated as the colonies grown to the initial culture concentration.

### 3.4.3. Determination of bile tolerance (Sahadeva *et al.*, 2011)

The mean intestinal bile concentration is believed to be 0.3% (w/v). Determination of bile salt tolerance was performed by pour plate and cup plate techniques. MRS agar media was supplemented with 0.2%, 0.3%, bile salts and inoculated with  $10^6$  CFU/ml from active capsule cell cultures and incubated according to the method followed.

**Pour plate technique:** MRS agar media was inoculated with  $10^6$  CFU/ml cells from active marketed probiotic sample in two plates and one plate was supplemented with 0.3% bile salt with quantity of 25 $\mu$ l and other plate was supplemented with 0.3% bile salt with the quantity of 100 $\mu$ l. After inoculation the media was poured on to the petriplates and incubated for 24h at 37° C along with the control plate.

**Cup plate technique:** The active marketed probiotic sample was inoculated to the sterile MRS agar medium, mixed thoroughly, and poured into sterile Petri plates and allowed to solidified. Cups were made using sterile stainless steel borer and 25 $\mu$ l of 0.2%(25 $\mu$ g/l,100 $\mu$ g/l) and 0.3%(25 $\mu$ g/l,100 $\mu$ g/l) bile salt solution was added. Then MRS agar plates were incubated for 24h at 37 °C.

## RESULTS AND DISCUSSION

### 4.1. Probiotic Properties of Actinomycete Isolate

#### 4.1.1. Determination of acid tolerance

Being resistant to low pH is one of the major selection criteria for probiotic strains. Since, to reach the small intestine they have to pass through from the stressful conditions of stomach.

Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below. As the existing actinomycete isolate was tested for their ability to tolerate the low pH, it was observed that there was no change in the growth of the inoculated organism.



Fig. 4.1: Isolate VMS-30 tested at pH 3. Fig. 4.2: Control.

#### 4.1.2. Determination of bile tolerance

- The strain, was tested for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v.
- Strains were detected in 0.2%, 0.3%, 0.4%, 0.5% bile salt concentration. The cfu values observed. According to the results VMS-30 strains were resistant to 0.2%, 0.3%, 0.4%, 0.5% bile salt. The isolate was able to grow in bile salt as they survive. Figures are shown below.

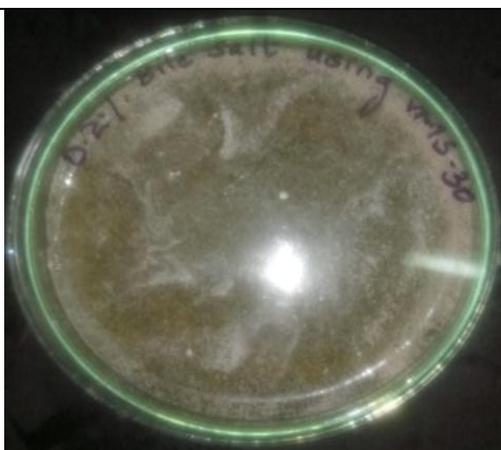


Fig. 4.3: Isolate VMS-30 tested at 0.2% bile salt. Fig. 4.4: Isolate VMS-30 tested 0.3% bile salt.



Fig. 4.5: Isolate VMS-30 tested at 0.4% bile salt. Fig. 4.6: Isolate VMS-30 tested at 0.5% bile salt.



Fig. 4.7: Control.

#### 4.1.3. Antimicrobial activity

- As the VMS-30 isolate was selected based on their antimicrobial activity.
- Those isolate was tested against indicator microorganisms *Staphylococcus aureus* and *Escherichia coli*.
- The isolate was shown the inhibition zone diameters against the indicator microorganisms, which indicates the isolate has the antibacterial activity.

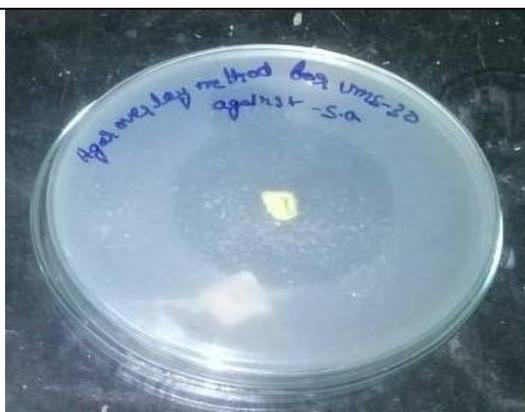


Fig. 4.8: Isolate VMS-30 tested against *S.aureus*

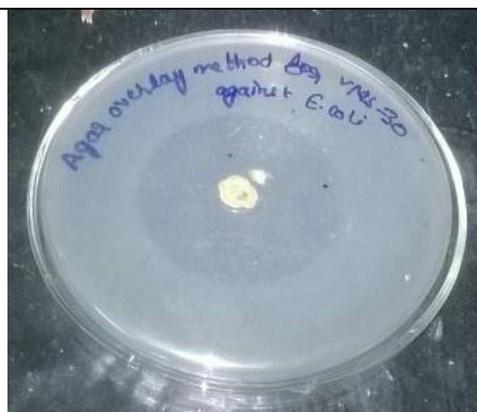


Fig. 4.9: Isolate VMS-30 tested against *E.coli*.

## 4.2. Evaluation of Probiotic Capsule

### 4.2.1. Determination of acid tolerance

Being resistant to low pH is one of the major selection criteria for probiotic strains. Since, to reach the small intestine they have to pass through from the stressful conditions of stomach. Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below. As the marketed probiotic sample was tested for their ability to tolerate the low pH, it was observed that there was increase in the growth of the microorganism, which indicates that the marketed probiotic sample was tolerate to the low pH.

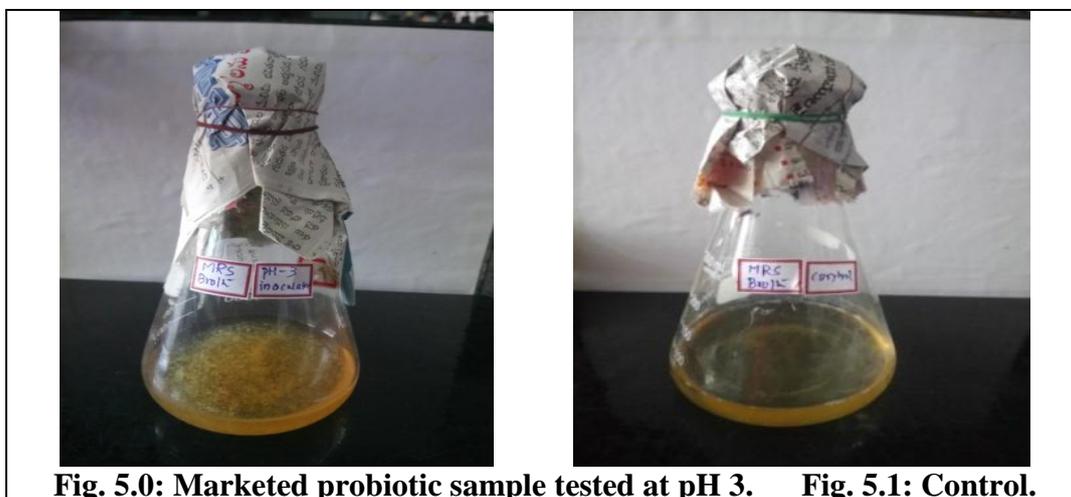


Fig. 5.0: Marketed probiotic sample tested at pH 3. Fig. 5.1: Control.

### 4.2.2. Determination of bile tolerance

The capsule cells, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v.

**Pour plate technique:** the capsule cells were tested for their ability to tolerate against 0.3% bile salt with the quantity of 25 $\mu$ l and 100 $\mu$ l. According to the results the capsule cells are resistant to 0.3% bile salt. figures are shown below

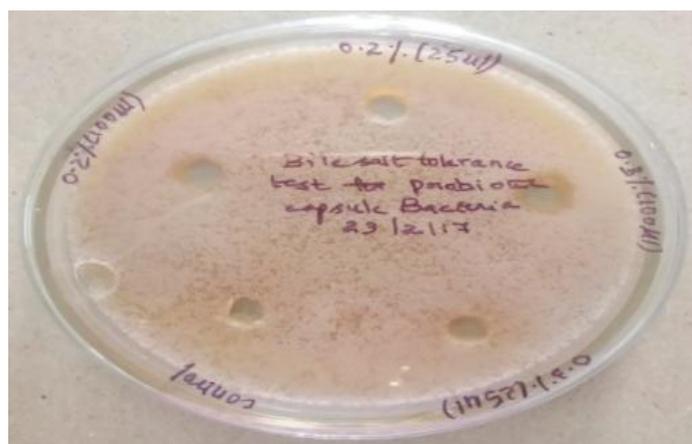


**Fig. 5.2:** Marketed probiotic sample tested against 0.3% bile salt (25µl).



**Fig. 5.3:** Marketed probiotic sample tested against 0.3% bile salt (100µl).

**Cup plate technique:** the capsule cells were tested for their ability to tolerate against 0.2%, 0.3% bile salt with the quantity of 25µl and 100µl. According to the results the capsule cells are resistant to 0.2% 0.3% bile salt. figures are shown below.



**Fig. 5.4:** Marketed probiotic sample tested against 0.2%, 0.3% bile salt (25µl, 100µl).



**Fig. 5.5:** Control.

## CONCLUSION

Among the tested cultures for probiotic activity VMS-30 showed bile salt tolerance at 0.2%, 0.3%, 0.4%, 0.5% bile salt. But in the case of acid tolerance the isolate VMS-30 showed no growth at pH 3, But in viable count method very few (two or three) colonies were observed. It also has good antibacterial activity towards the indicator microorganisms. Whereas the marketed sample which was evaluated for their ability towards low pH and bile salt tolerance, the marketed probiotic sample had shown good acid tolerance and bile salt tolerance. As per the section criteria of probiotics, resistance towards low pH which act as main probiotic property, As our isolate was sensitive to low pH, it may require further genetic modifications for their ability to tolerate the low pH.

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