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# *Bacillus velezensis* BNC -1, a Novel Salt-Tolerant Biocontrol Agent Obtained from Salted *Hilsa* Fish Prohibits the Growth of Vegetable/Fruit Pathogen

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**Abstract:** Anti-metabolites produced by microorganisms, particularly bacteria and fungi, are an essential biochemical with the highest potential for application as a bio-preservative agent because they have antibacterial capabilities and have no human adverse effects. The literature search found that just a little amount of study has been done thus far. Bio-preservation is a potential approach for reducing the risk of deterioration in the food industry. The proposed study's goals were to identify antimicrobial/anti-metabolite-producing microorganisms from natural sources and investigate their antibacterial effectiveness against food spoilage microorganisms. A total of 76 bacterial cultures were recovered from diverse dietary samples during the investigation. All of these isolates were purified and tested for antibacterial activity against bacterial cultures and fungi isolated from rotten vegetables/fruits, among other things. MRS medium was used to keep the isolates alive. Fungi isolated from several spoiled vegetables were purified and maintained in Czapec-Dox agar media. The antimicrobial activity of the supernatant from the isolates after 18 h of incubation was tested against the pathogenic fungi isolated from rotten vegetable Ivy gourd (*Coccinia grandis*) and rotten fruits -- Lemon (*Citrus limon*), Pear (*Pyrus*), and Banana (*Musa*) by paper disk assay method. Three isolates, IN-1 (isolated from rotten naspati), IPM (isolated from rotten palm product), and ISF-1 (isolated from salted fish), were found to have the most potent antimicrobial activity against standard bacterial cultures and fungi isolated from spoiled fruits, out of a total of seventy-six isolates. The antimicrobial activity of the supernatant was evidenced by the clear zone of inhibition ranging from 19-22 mm by using 50 µl of soup. Based on morphological, physiological, and biochemical properties, one of the bacteria, ISF-1, was thoroughly characterized. ISF-1 was identified as *Bacillus velezensis* by a 16S rRNA sequencing study, having 54.985755 % G-C nucleotide base pair.

**Keywords:** Biocontrol, *Bacillus velezensis* , Rotten food, Vegetables, Bacteria, Fungi

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## Introduction

Many food products are naturally perishable, thus in order to preserve their stipulated shelf life during preparation, storage, and distribution, they must be guarded against rotting. The demand for minimally processed, easily prepared, and ready-to-eat fresh food products, as well as the globalization of food trade and distribution from centralised production, are all harming food safety and quality. Food products can be contaminated by fungi and bacteria. A lot of these bacteria have the potential to negatively affect food, leading it to lose its flavour, odour, colour, sensory, and textural features.

Microbial proliferation is a significant issue because some microorganisms have the potential to cause food-borne diseases. In packaged foods, intrinsic factors like pH and oxygen, as well as extrinsic factors like temperature, time, and relative humidity, all have an impact on pathogens like *Listeria monocytogene*s, *Escherichia* coli O157, *Salmonella, Staphylococcus aureus, Bacillus cereus, Campylobacter, Clostridium perfringens, Aspergillus niger, and Saccharomyces cerevisiae*. The food industry has used a number of preservation techniques, such as heat treatment, salting, acidification, and drying, to prevent food from spoiling and pathogenic bacteria from growing (Davidson and Taylor, 2007).

There have been numerous initiatives to identify natural alternatives to inhibit bacterial and fungal growth in meals. Foods preserved with natural chemicals have grown highly popular in recent years as a result of increased consumer awareness and concern about synthetic chemical additives.

Antimicrobials can be directly added to the product composition, coated on its surface, or included in the packaging material to hinder the growth of unwanted microbes in food. Antimicrobial films can continue their action for a long time, whereas direct inclusion of active chemicals into food results in an instantaneous but short-term reduction of bacterial populations (Appendini and Hotchkiss, 2002; Hanušová *et al.*, 2009). Essential oils from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes from animals (e.g., lysozyme, lactoferrin), bacteriocins from bacteria (nisin, natamycin), organic acids (e.g., sorbic, propionic, citric acid), and naturally occurring polymers are the most common natural compounds (chitosan). Plant essential oils, which are generally recognized as safe, are garnering a lot of attention in the food business because of their potential as decontaminating agents (GRAS). The active ingredients are widely found in essential oil fractions, and it is well known that most of them exhibit antibacterial activity against food-borne diseases and spoilage microorganisms (Gutierrez *et al.*, 2008).

The presence of hydrophilic functional groups, such as hydroxyl groups of phenolic components and/or lipophilicity of certain essential oil components, contributes to the antibacterial action of plant essential oils (Dorman and Deans, 2000). The most efficient substances are those having phenolic groups, such as clove, oregano, rosemary, thyme, sage, and vanillin oils (Skandamis *et al.*, 2002). Gram-positive bacteria are more resistant to them than Gram-negative bacteria (Mangena and Muyima, 1999; Marino *et al.*, 2001) .

The main antibacterial component of mustard and horseradish oil is allyl-isothiocyanate. It works better against Gram-negative bacteria and has little to no effect on lactic acid bacteria. Although the volatile compound's antibacterial effectiveness varies (Delaquis and Mazza, 1995), it is particularly effective against *E. coli* (Nadarajah *et al.*, 2002; Muthukumarasamy *et al.*, 2003).

The utilization of bacteriocin-producing lactic acid bacteria or their more or less purified bacteriocins has also gotten a lot of attention. Bacteriocins are tiny bacterial peptides with potent antibacterial activity against bacteria that are closely related. *Lactococcus lactis* spp. produces nisin, a polypeptide. It has been approved as a GRAS food additive in more than 50

countries throughout the world. It has a wide range of effectiveness against lactic acid bacteria as well as other Gram-positive bacteria. Furthermore, it is highly efficient against heat-resistant *Clostridium botulinum* bacterial spores as well as food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* (Brewer *et al.*, 2002; Lopez-Pedemonte *et al.*, 2003; Sobrino-Lopez and Martin-Belloso, 2006).

The combination of nisin and ethylenediamine tetra-acetic acid (EDTA) may improve the effectiveness. Furthermore, exposure to chelating chemicals, sub-lethal heat, osmotic shock, and freezing can improve the action of nisin since these treatments render Gram-negative microbes' cell walls more permeable and hence more sensitive to the nisin (Gálvez *et al.*, 2007).

Another group of natural substances that have been used in food as effective preservatives are enzymes. Lysozyme, for example, is a lytic enzyme that can hydrolyze -1,4 bonds between N-acetylmuramic acid and N-acetyl glucosamine and is found in foods like milk and eggs (Cunningham *et al.*, 1991). Lysozyme has been used commercially to prevent *Clostridium tyrobutyricum* from causing late blowing in semi-hard cheeses. Because Gram-negative bacteria have a lipopolysaccharide layer in their outer membrane, lysozyme is bactericidal against Gram-positive bacteria but ineffective against Gram-negative bacteria. Since the 1960s, it has been known that using membrane-disrupting chemicals such as detergents and chelators (EDTA) might increase Gram-negative bacteria's susceptibility to lysozyme lysis (Vara, 1992; Shelef and Seiter, 1993; Branen and Davidson, 2004)

Because their efficacy is well-recognized and cost-effective, organic acids and their salts are routinely employed as chemical antibacterial agents. Acetic, lactic, propionic, sorbic, and benzoic acid are the most potent organic chemicals. The rise in proton concentration, which lowers the external pH, has an antibacterial impact. Organic acids can cause bactericidal effects by affecting the integrity of microbial cell membranes or cell macromolecules, as well as interfering with nutrient transport and energy metabolism (Ricke, 2003).

Organic acid production had been conceivable before the discovery of microbes, with lactic acid being commercially manufactured via fermentation for the first time in 1880, but the bulk of organic acids was chemically extracted or synthesized from other compounds. Mixtures of acids which could exert a wider antimicrobial activity than a single organic acid (Theron *et al.*, 2010).

Chitosan, a natural antibacterial, has attracted a lot of attention for commercial applications. Because of its great biodegradability and antibacterial characteristics, it has been employed in the medicinal, food, agricultural, and chemical industries. Chitosan's biological activity is determined by its molecular weight, degree of deacetylation, and derivatization, as well as the degree of substitution, length, and position of a substitute in chitosan's glucosamine units, pH of the chitosan solution, and the target organisms (No *et al.*, 2007). It has made commercially from crab and shrimp shell wastes, and it comes in a variety of deacetylation grades and molecular weights. As a result, it has a variety of functional features, including emulsification, dye binding, and gelation. Chitosan has also been documented to possess a film-forming property for use as edible film or coating, to decrease water vapor and oxygen transmission, diminish respiration rate and increase the shelf life of fruit (Jiang and Li, 2001).

The present study was aimed to identify antimicrobial/anti-metabolite-producing microorganisms from natural sources and investigate their antibacterial effectiveness against food spoilage microorganisms.

## Materials and Methods

*Isolation of anti-metabolite-producing organisms/bacteria from natural sources by crowded plate technique*:

To begin, several samples (rotten food materials such as milk, spoiled fish, spoiled meat product, idli, dosa, and so on) were acquired from the local market. In a test tube, one gram of each sample was placed and suspended in 10 ml sterile distilled water. This mixture was then used to inoculate and spread on MRS agar medium, which was subsequently incubated for 15 days at 37℃. After incubation, colonies were developed on MRS agar plates. Colonies surrounded by clear zone were separated and kept as pure cultures on MRS agar slants.

*Screening for antimicrobial activity against standard bacterial cultures*:

Colonies grown on MRS agar slants were inoculated with MRS broth and stored overnight at 37°C in an incubator. After incubation, 1.5 ml of each separated organism's broth culture was transferred to a sanitized Eppendorf tube and centrifuged at 10,000 rpm for 15 min. After centrifugation, 50 µl cell-free culture supernatant was poured into wells on agar plates that had previously been inoculated with indicator organisms (MTCC 9498, MTCC 903, and MTCC 3041) and incubated at 37°C for 24 h. After incubation, plates were observed for the presence of a zone of inhibition. Among the seventy-six isolates, bacteria isolated from salted fish (ISF-1), rotten naspati (IN-1), and rotten palm product (IPM) showed antimicrobial activity against the three indicator organisms. Diameters of zones of inhibition were recorded.

*Isolation of food spoilage fungi from different spoiled fruits and screening of their sensitivity by well diffusion method*:

From the local market, decaying vegetables like Ivy gourd (*Coccinia grandis*) and rotten fruits like Lemon (*Citrus limon*), Pear (*Pyrus*), and Banana (*Musa*) were obtained. In a test tube, 1 g of each sample was placed and suspended in 10 ml of sterile distilled water. After that, the mixture was steaked on a Czapec-Dox agar plate and incubated for 3-4 days at 37°C. Fungi found on Czapec-Dox agar plates were isolated and grown as a pure culture on Czapec-Dox agar slants.

ISF-1, IN-1, and IPM were next tested for antibacterial efficacy against fungi isolated from damaged vegetables and fruits. A 50 µl aliquot of ISF-1, IN-1, and IPM cell-free culture supernatant was added to the wells of Czapexdox agar plates infected with fungus recovered from damaged fruits. Clear zones of inhibition were visible around the wells after 7 days of incubation at 37°C. The diameters of inhibitory zones were measured.

*Morphological and biochemical characterization of ISF-1 and IN-1*:

Gram staining was used to assess the Gram character of ISF-I and IN-1 isolates. Isolates were grown in MRS broth for 24 and 48 h at varying temperatures (4°C, 10°C, 30°C, 37°C, and 45°C), pH (3, 5, 7, and 9), and salt (NaCl) concentrations (5 per cent, 10 per cent, 15 per cent, and 20 per cent) to determine their optimal growth temperature, pH, and salt tolerance.

The ability of isolates to use different sugars as their sole carbon source was also examined by growing them in MRS broth containing different sugars (Maltose, Inositol, Trehalose, Dextrose, Galactose, Fructose, Cellobiose, Rhamnose, Lactose, Sucrose, and Mannitol). ISF-1 and IN-1 were inoculated in 250 ml MRS broth medium and incubated in a rotary shaker at 37°C to determine the growth curve. The absorbance of the culture was measured in a colorimeter at 540 nm at 1 h intervals. 16S rRNA sequencing identified ISF-1 as *Bacillus velezensis* strain BNC-1.

## Results and Discussion

*Screening for antimicrobial activity against standard bacterial cultures and fungi isolated from spoiled fruits*:

Using the crowded plate approach, 76 morphologically distinct bacterial colonies were picked from various food samples and pure cultured. The paper disc method was used to test each isolated strain for antagonistic activity against standard bacterial cultures (Figs. 1-7). Only ISF-1, IN-1, and IPM were found to have antibacterial action against conventional bacterial

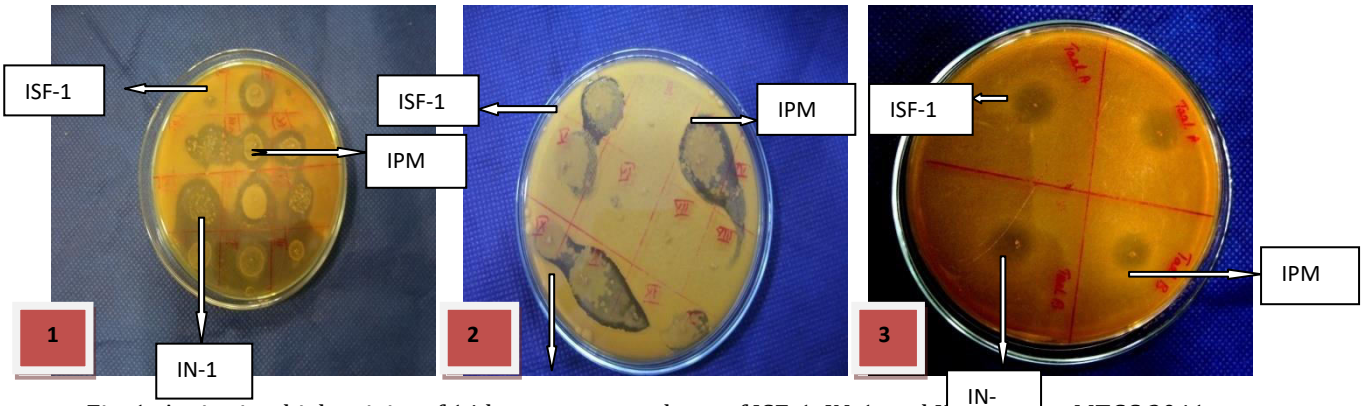


Fig. 1: Anti-microbial activity of 14 hours grown culture of ISF-1, IN-1, and I st MTCC 3041.
Fig. 2: Anti-microbial activity of 14 hours grown culture of ISF-1, IN-1, and IPM against MTCC 903.
Fig. 3: Anti-microbial activity of 14 hours grown culture of ISF-1, IN-1, and IPM against MTCC 9498.

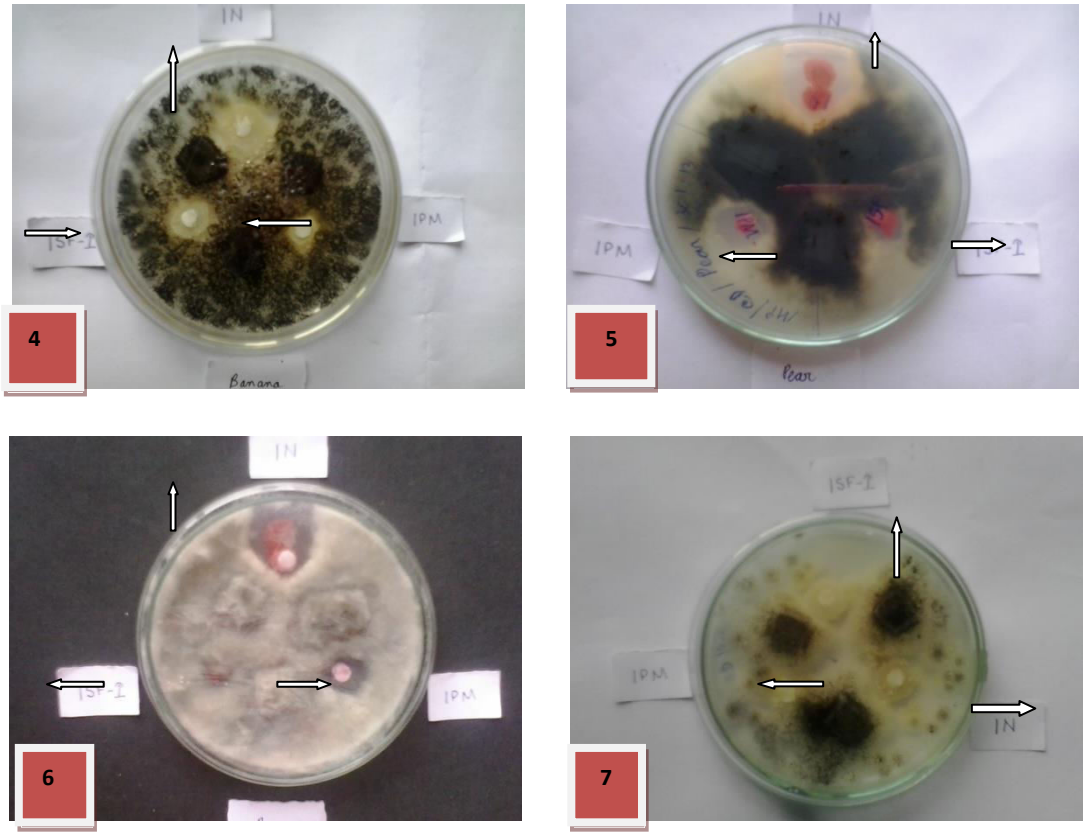


Fig. 4: Anti-microbial activity of three isolates against fungi isolated from rotten Ivy gourd (FII).
Fig. 5: Anti-microbial activity of three isolates against fungi isolated from rotten Lemon (FIL).
Fig. 6: Anti-microbial activity of three isolates against fungi isolated from rotten Pear (FIP).
Fig. 7: Anti-microbial activity of three isolates against fungi isolated from rotten Banana (FIB).

Table 1: Morphological characteristics of ISF-1, IN-1, and IPM

| Isolates | Colony characters | | | | |
|---|---|---|---|---|---|
| | Form | Elevation | Margin | Surface appearance | consistency |
| ISF-I | irregular | raised | undulate | wrinkled | rubbery |
| IPM | circular | raised | Lobate, hilly | Wrinkled | sticky |
| IN | irregular | flat | undulate | wrinkled | Sticky |

Table 2: Physical characteristics of ISF-1 and IN-1

| Sample | Parameter | Variation | Growth after 24 h | Growth after 48 h |
|---|---|---|---|---|
| IN-1 | Temperature | 4°C | - | - |
| | | 10°C | - | - |
| | | 30°C | + | ++ |
| | | 37°C | ++ | +++ |
| | | 45°C | + | ++ |
| | pH | 3 | - | - |
| | | 5 | - | + |
| | | 7 | + | + |
| | | 9 | - | - |
| | Salt Concentration | 5% | - | + |
| | | 10% | - | + |
| | | 15% | - | +++ |
| | | 20% | - | - |
| ISF-1 | Temperature | 4°C | - | - |
| | | 10°C | - | + |
| | | 30°C | + | ++ |
| | | 37°C | ++ | +++ |
| | | 45°C | + | ++ |
| | pH | 3 | - | - |
| | | 5 | - | + |
| | | 7 | + | + |
| | | 9 | - | - |
| | Salt Concentration | 5% | - | +++ |
| | | 10% | - | +++ |
| | | 15% | - | ++ |
| | | 20% | - | - |

cultures (MTCC 9498, MTCC 903, and MTCC 3041) as well as fungi isolated from rotten Ivy gourd (FII), rotten Banana (FIB), rotten Lemon (FIL), and rotten Pear (FIP). IN-1 had the most antimicrobial activity of the three isolates when tested against conventional bacterial cultures and fungi obtained from decaying fruits.

*Morphological, Physiological, and Biochemical characteristics of ISF-1 and IN-1*:

ISF-1 and IN-1 were characterized morphologically (Table 1) and biochemically using 24-hour-old cultures. Both ISF-I and IN-1 are gram-positive rod-shaped bacteria that live in chains, according to gram staining and microscopic examination.

Growing the isolates ISF-1 and IN-1 at different temperatures (4°C, 10°C, 30°C, 37°C, and 45°C), (Table 2) at different pH (3, 5, 7, and 9), and

Table 3: Sugar utilization of ISF-1 and IN-1

| Carbon sources | ISF -1 | IN-1 |
|---|---|---|
| | After 24 h | After 24 h |
| Maltose | + | + |
| Inositol | + | + |
| Trehalose | + | + |
| Dextrose | + | + |
| Galactose | + | + |
| Fructose | + | + |
| Cellobiose | - | - |
| Rhamnose | + | + |
| Lactose | + | + |
| Sucrose | + | + |
| Mannitol | + | - |

Table 4: OD Details

| Incubation Period (h) | OD | | | | | |
|---|---|---|---|---|---|---|
| | 0% NaCl concentration | 2% NaCl concentration | 4% NaCl concentration | 6% NaCl concentration | 8% NaCl concentration | 10% NaCl concentration |
| 3 | 0.05 | 0.02 | 0 | 0 | 0 | 0 |
| 6 | 0.4 | 0.2 | 0.02 | 0.02 | 0 | 0 |
| 9 | 0.8 | 0.37 | 0.03 | 0.05 | 0 | 0 |
| 12 | 1.1 | 0.6 | 0.06 | 0.08 | 0.01 | 0 |
| 15 | 1.3 | 0.82 | 0.2 | 0.12 | 0.02 | 0 |
| 18 | 1.4 | 1.1 | 0.4 | 0.24 | 0.03 | 0.01 |
| 21 | 1.5 | 1.25 | 0.7 | 0.4 | 0.05 | 0.02 |
| 24 | 1.6 | 1.3 | 0.9 | 0.58 | 0.07 | .02 |
| 27 | 1.55 | 1.4 | 1.2 | 0.85 | 0.15 | .02 |
| 30 | 1.5 | 1.35 | 1.2 | 1.1 | 0.4 | .02 |
| 33 | 1.4 | 1.3 | 1.15 | 1.2 | 0.8 | .02 |

different salt concentrations (5 per cent, 10 per cent, 15 per cent, and 20 per cent) (Table 2) and then analyzing the turbidity of the medium after incubation revealed that both ISF-1 and IN-1 were able to grow at 30°C, 37°C, and 45°C, but only at 37°C.

Only the isolates ISF-1 and IN-1 were able to develop at pH 7, but not at pH 3, pH 5, or pH 9. Isolates ISF-1 and IN-1 may withstand 5-15 per cent sodium chloride (NaCl) but not more than 20 per cent. ISF-1 can grow at a maximum of 5-10% salt, while IN-1 can grow at a maximum of 15% salt. The ability of isolates to use different sugars as their sole carbon source was also examined by growing them in MRS broth containing different sugars (Maltose, Inositol, Trehalose, Dextrose, Galactose, Fructose, Cellobiose, Rhamnose, Lactose, Sucrose, and Mannitol). ISF-1 was able to use all sugars except cellobiose, while IN-1 was able to use all sugars except cellobiose and mannitol (Table 3).

ISF-1 has a lag phase of 0-4 h, a log phase of 4-11 h, an exponential phase of 11-18 h, and a death phase that begins after 18 h, according to its

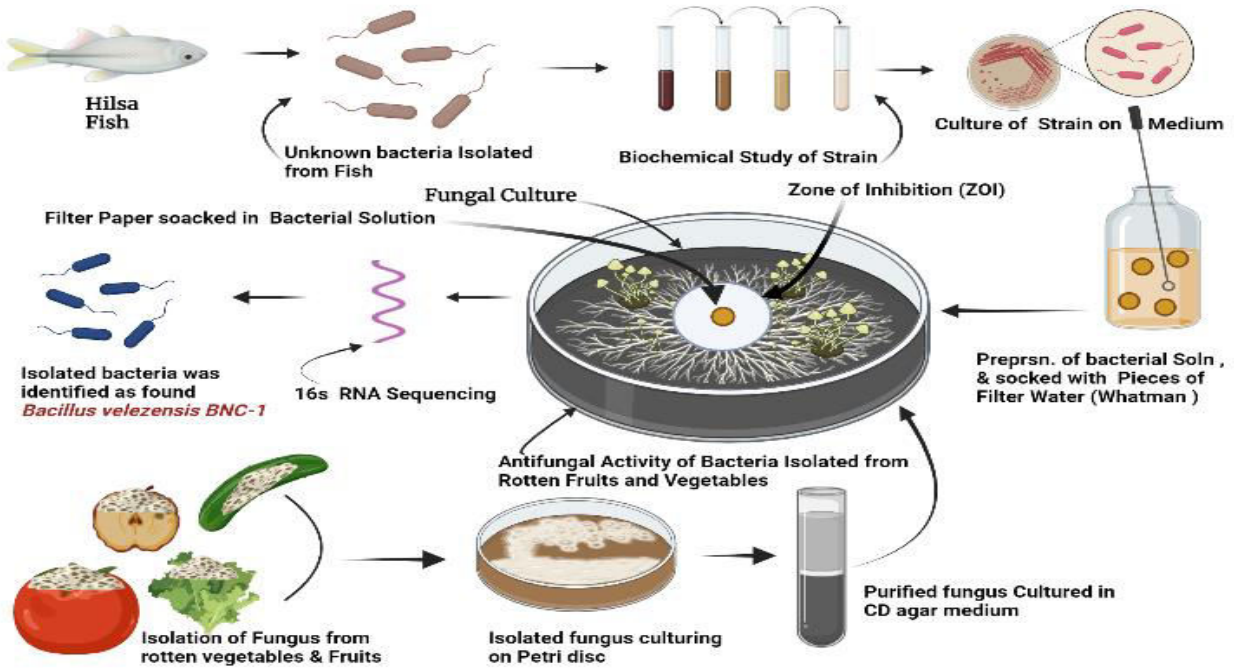


Fig. 8: Diagrammatic representation of current research.

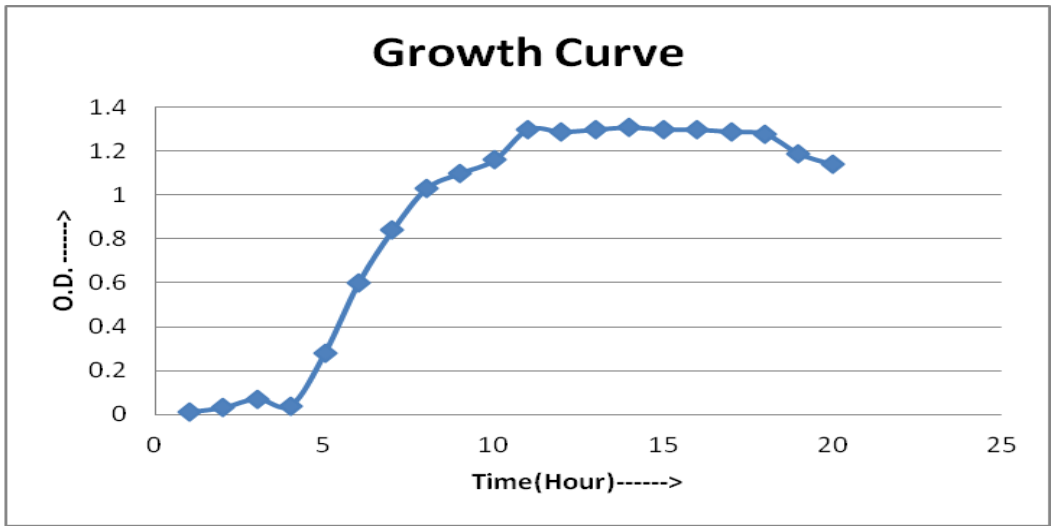


Fig. 9: Growth Curve of IN-1 isolated from rotten Naspati in terms of OD at different time interval.

development curve. IN-1 has a lag phase of 0-4 h, a log phase of 4-10 h, an exponential phase of 10-16 h, and a death phase that begins after 16 h, according to its growth curve with angle.

*OD* (*Optical Density*):

Monitoring bacterial cell growth and analysing their physical characteristics is crucial for identifying the likelihood of a bacterial infection as well as for better comprehending the circumstances in which these organisms survive and subsequently proliferate. This is crucial to use or inoculate known quantities of the bacteria, say to boost plant growth, accelerate the breakdown of harmful chemical compounds, or industrially create antibiotics or other natural products. For

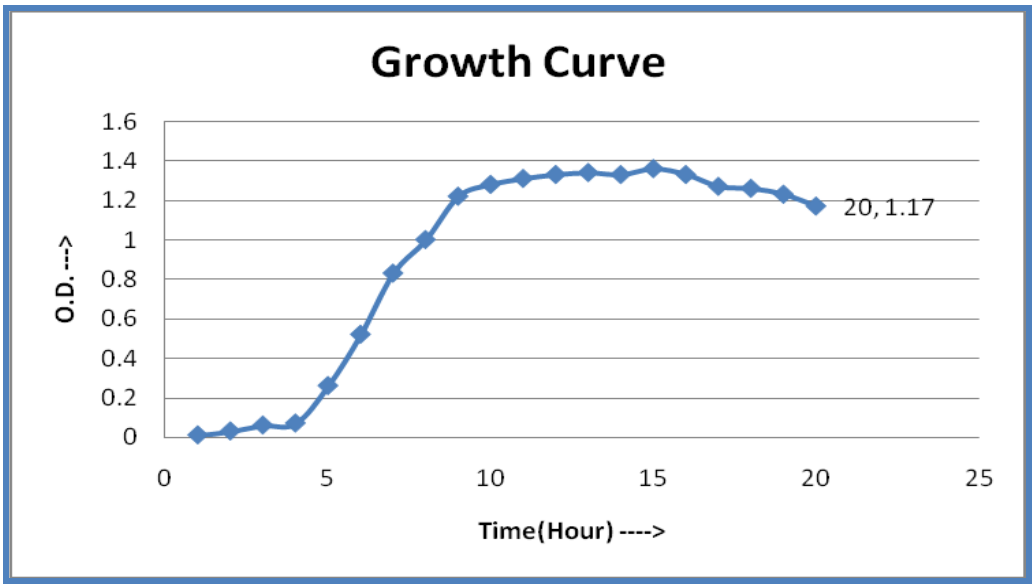


Fig. 10: Growth Curve of ISF-1 isolated from salted *Hilsa* fish in terms of OD at different time interval.

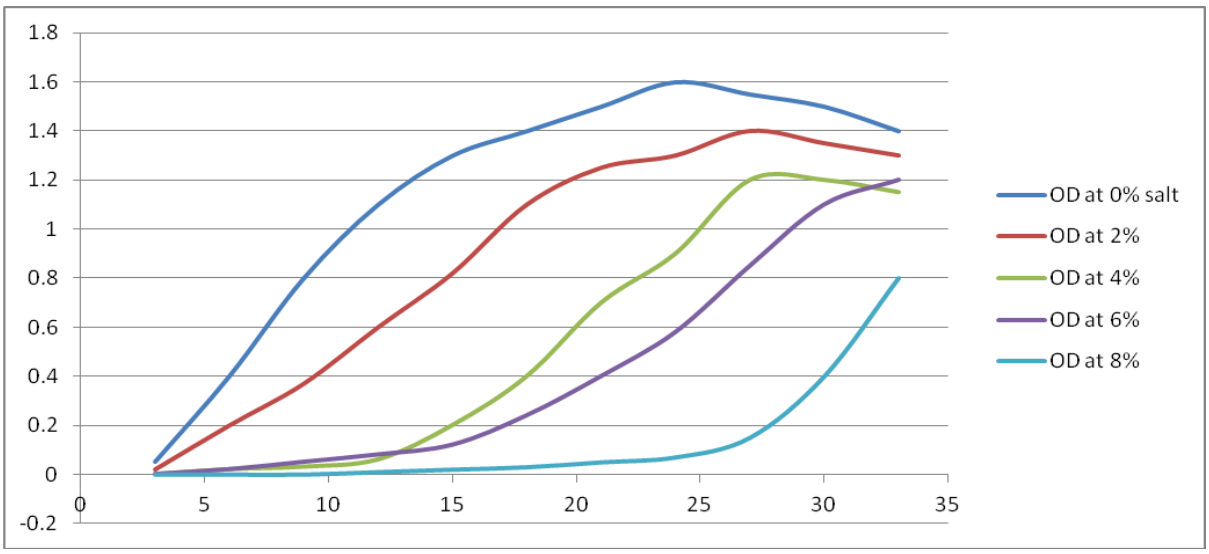


Fig. 11: Growth Curve of ISF-1 in presence of graded concentration of Salt Concentration (5).

the current research, OD details are given against concentrations of NACL (0%), (2%), (4%), (6%), (8%) and (10%) for the incubation periods (3 h), (6 h), (9 h), (12 h), (15 h), (18 h), (21 h), (24 h), (27 h), (30 h) and (33 h) (Table 4). The OD curve samples i.e. ISF-1 and IN -1 has been depicted in Figures 8 and 9.

*Growth curve of ISF 1*:

The growth curve was prepared by inoculating ISF-1 bacteria in MRS broth medium and OD was measured at 540 nm at regular time interval upto 20 hrs. From the result it was observed that ISF-1 has a lag phase of 0-4 h, a log phase of 4-11 h, an exponential phase of 11-18 h, and a death phase that begins after 18 h, according to its growth curve.

*Growth curve of IN-1*:

The growth curve of IN-1 was prepared by inoculating bacteria in MRS broth medium and OD was measured at 540 nm at regular time interval

upto 20 hrs IN-1 has a lag phase of 0-4 h, a log phase of 4-10 h, an exponential phase of 10-16 h, and a death phase that begins after 16 h, according to its growth curve with angle.

## Conclusion

Three isolates, IN-1 (isolated from rotten naspati), IPM (isolated from rotten palm product), and ISF-1 (isolated from salted fish), were found to have the most potent antimicrobial activity against standard bacterial cultures and fungi isolated from spoiled fruits, out of a total of seventy-six isolates. The antimicrobial activity of the supernatant was evidenced by the clear zone of inhibition ranging from 19-22 mm by using 50 µl of soup. Based on morphological, physiological, and biochemical properties, one of the bacteria, ISF-1, was thoroughly characterized. ISF-1 was identified as *Bacillus velezensis* by a 16S rRNA sequencing study, having 54.985755 % as G-C nucleotide base pair.

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