

BIOCONTROL OF FRUIT /VEGETABLE PATHOGENS BY BACTERIAL ISOLATES/CELL FREE EXTRACT ISOLATED FROM DEGRADED FRUIT/FOOD PRODUCTS

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ABSTRACT

Anti-metabolites produced from microorganisms specially bacteria and fungi are the important biochemical which has the greatest potential to be used as bio-preservative agent as it has anti microbial property as well as no side effects on human. From literature search it was revealed that there are only limited work has been done so far. Bio-preservation a promising strategy to control spoilage risk against lightly preserved food industries. The objectives of the proposed research are to isolate some antimicrobial / anti-metabolite producing microorganism from natural sources and study their antimicrobial activity against food spoiling microorganisms. During study a total twenty six bacterial cultures were isolated from various food samples. All these isolates were purified and screened for their antimicrobial activity against some standard bacterial cultures and fungi isolated from spoiled vegetables/fruits etc. Among twenty six isolates, 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 most potent for anti-microbial activity against standard bacterial cultures and fungi isolated from spoiled fruits. The isolates were maintained on MRS medium. The organism is fast growing mesophile with low generation time. Fungi isolated from several spoiled vegetables were purified and maintained in Czapek-Dox agar media. The antimicrobial activity of the supernatant from the isolates after 18 hours of incubation was tested against the pathogenic fungi isolated from Rotten vegetable like Kundri (Eng. Ivy guard) (*Coccinia grandis*), Bitter guard (*Momordica charantia*), green potato (*Solanum tuberosum*) and rotten fruits like Lemon (*Citrus limon*), Pear (*Pyrus*) and Mango (*Mangifera indica*) by paper disk assay method. The antimicrobial activity of the supernatant was evidenced by the clear zone of inhibition ranging from 1.8-3.6 cm by using 50 µl soup.

KEYWORDS: *Solanum tuberosum*, *Momordica charantia*, *Coccinia grandis*, Biocontrol, pathogenic fungi.

INTRODUCTION

Perishable food products require protection from spoilage during their preparation, storage, and distribution. The demand for processed fresh food products, globalization of food trade, and distribution from multinational companies processing pose major challenges for food safety and quality. Food products can be subjected to contamination by microbes. Many of these microorganisms can cause undesirable reactions that deteriorate flavor, odor, color, sensory, and textural properties of foods. Microbial growth is a major threat because some microorganisms can potentially cause food poisoning by secreting toxins. In packaged foods, growth and survival of common spoilage and pathogenic microorganisms such as *Listeria monocytogenes*, *Escherichia coli* O157, *Salmonella*, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter*, *Clostridium perfringens*, *Aspergillus niger*, and *Saccharomyces*

cerevisiae are affected by a variety of intrinsic factors, such as presence of oxygen and pH or by extrinsic factors associated with storage conditions including relative humidity, time and temperature.^[1,2,3]

To prevent growth of spoilage and pathogenic microorganisms in foods, several physical and chemical preservation techniques, such as heat treatment, salting, acidification, and drying have been applied in the food industry.^[4,5] Benzoic acid, sulphur dioxide, sodium nitrite, sorbates, ethylene diamine tetra acetic acid, citric acid and butylated hydroxytoluene (BHT) are some of the examples of synthetic preservatives commonly used for enhancing the shelf life of edible materials, but their full compatibility with the human system is still questionable. Moreover, sulphur dioxide causes breathing difficulties; sodium nitrite^[6] and BHT^[7] are reported to be carcinogenic. In recent years concern regarding synthetic chemicals, additives have been

increased because of the great consumer awareness and foods preserved with natural additives have become very popular. Natural preservatives are thought to be better alternatives. Traditionally, herbal drugs were utilized in the form of mixture of different plants. Plant-based preservatives are biodegradable, renewable, and safe for non-target organisms and have diverse biological effects. They provide less chance of resistance development to microbes. To control undesirable microorganisms in food, the antimicrobials can be directly added into the product formulation, coated on its surface or incorporated into the packaging material. Direct incorporations of active agents into food result in an immediate but short-term reduction of bacterial populations, while the antimicrobial films can maintain their activity for a long period of time.^[8,9]

Main natural compounds screened for food preservations are essential oils derived from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes obtained from animal sources (e.g., lysozyme, lactoferrin), bacteriocins from microbial sources (nisin, natamycin), organic acids (e.g., sorbic, propionic, citric acid) and naturally occurring polymers (chitosan). In this context, plant essential oils are gaining a wide interest in food industry for their potential as decontaminating agents, as they are Generally Recognized as Safe (GRAS). The active components are commonly found in the essential oil fractions and it is well established that most of them have a wide spectrum of antimicrobial activity, against food-borne pathogens and spoilage bacteria.^[10,11]

The use of bacteriocin-producing lactic acid bacteria or their more or less purified bacteriocins has been also receiving increased interest. Bacteriocins are small bacterial peptides that show strong antimicrobial activity against closely related bacteria. Bacteriocin is relatively broad spectrum in activity against different lactic acid bacteria and other gram positive bacteria. It has been approved as a food additive with GRAS status in over 50 countries worldwide.^[12,13,14,15]

Modern life conditions in consequence of globalization, contribute to the major incidence of food disease outbreaks. Infectious intestinal diseases caused by bacteria, viruses, or parasites continue to be a major source of public health concern, social and economic costs worldwide. Listeriosis was reported from ready to eat (RTE) foods, mainly from fishery products and soft cheeses, while the highest proportion of Shiga toxin positive food samples was detected in meat from ruminants.^[16] In the USA, the reported food borne outbreaks predominantly involved the presence of *Salmonella* in raw vegetables, chicken/eggs, pork, and tuna. *Listeria (L.) monocytogenes* was detected from dairy products in raw beef, chicken salads, and raw vegetables.^[17] Biological preservation to ensure the hygienic quality of food has become a promising tool.^[18] From various studies scientists speculate that LAB can

be exploited as microbial cell factories and used in several applications such as biopreservation, shelf-life extension, fermentation biocontrol, human and veterinary medicine, and agriculture.^[19,20]

The main objective of the proposed investigation is to isolate the Antimicrobial substances producing Bacteria from spoiled food samples and purified them. The characters of the antimicrobial producing bacteria will be studied and its application for the controlling of fruit/vegetable pathogens in the laboratory condition will be analyzed. Pathogenic food fungi will also be isolated, purified and preserved to study the inhibitory effect of the antimicrobial producing bacteria against food pathogenic fungi.

From literature search it was revealed that there are only limited work has been done so far. Bio-preservation a promising strategy to control Spoilage risk against lightly preserved food industries.

MATERIALS AND METHODS

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

First different samples (spoiled food materials, e.g. milk product, spoiled fish, spoiled meat product, idli, dosa etc.) were collected from local market. One gram of each individual sample was taken in a test tube and suspended in 10 ml sterilized distilled water. This mixture was then used to inoculate and spread on MRS agar medium and incubated at 37°C for 3-7 days. Colonies were appeared on MRS agar plates after incubation. Colonies surrounded by clear zone were isolated and maintained on MRS agar slants as pure cultures.

Screening for antimicrobial activity against standard bacterial cultures

Colonies maintained on MRS agar slants were inoculated in MRS broth and kept in incubator for overnight at 37°C. After incubation 1.5 ml of broth culture of each isolated organisms was taken in sterilized eppendorf and centrifuged at 10,000 rpm for 15 mins. After centrifugation, 50 µl cell-free culture supernatant was applied into the well on agar plates previously inoculated with indicator organisms (MTCC 3041, *Lactococcus lactis subsp. lactis*) and then plates were incubated at 37°C for 24 hours. The indicator organism was procured from Microbial Type Culture Collection (MTCC), Chandigarh. After incubation plates were observed for the presence of zone of inhibition. Among the seventy six isolates, bacteria isolated from salted fish (ISF-1), rotten naspati (IN-1) and rotten palm product (IPM) were shown 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

Rotten vegetable/fruits like Mango (*Mangifera indica*), Lemon (*Citrus limon*), Kundri (Eng. Ivy guard) (*Coccinia grandis*), Pear (*Pyrus*), Bitter guard (*Momordica charantia*) and green potato (*Solanum tuberosum*) were collected from local market. One gram of each individual sample was taken in a test tube and suspended in 10 ml sterilized distilled water. This mixture was then streaked on Czapek-Dox agar plate and incubated at 37°C for 3-4 days. Fungi appeared on Czapek-Dox agar plates were then isolated and maintained as pure culture on Czapek-Dox agar slants.

MRS broth medium and Czapek-Dox agar medium were prepared in culture tubes containing 5ml media each. MRS broths were inoculated with the four test isolates and incubated under shaking condition for about 18 hours at 37°C. The supernatant of each sample was then collected by centrifugation at 10000 rpm for 10 min. Six Czapek-Dox agar plates were prepared and six fungal cultures were inoculated on six different plates. Four wells were made on each Czapek-Dox agar plate and filled with 50 ul supernatant of the four isolates. Then the plates were incubated at 37°C for 3 to 5 days. Observations were made and the effects of the supernatants on the spoilage organisms were recorded.

Morphological and biochemical characterization of ISF-1

For determination of growth curve, ISF-1 was inoculated in 100 ml MRS broth medium and incubated in a rotary shaker at 37°C. At 1 hour intervals, the absorbance of the culture was monitored in a colorimeter at 540 nm.

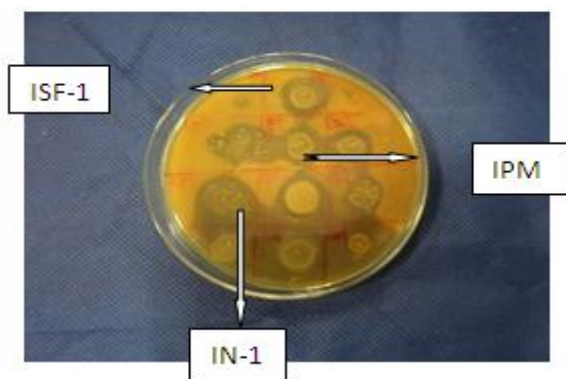
RESULTS AND DISCUSSION

Morphological, physiological and biochemical characters of ISF-1

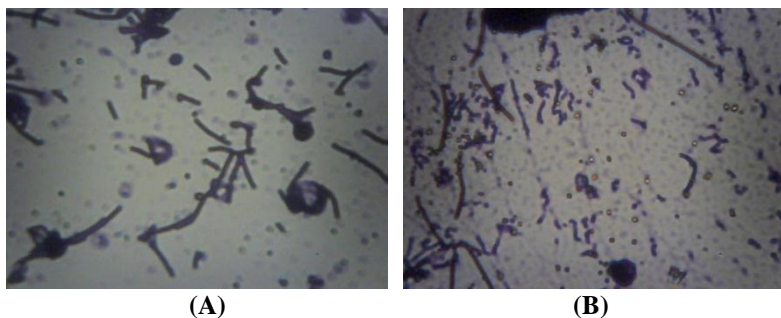
Growth curve of ISF-1 (Fig-1) showed that it has a lag phase between 0-4 hours, log phase between 4-11 hours, exponential phase between 11-18 hours and death phase starts after 18 hours. Growth curve of IN-1 (Fig-2) showed that it has a lag phase between 0-4 hours, log phase between 4-10 hours, exponential phase between 10-16 hours and death phase starts after 16 hours. By 16S rRNA sequencing analysis isolate ISF-1 was identified as *Bacillus methylotropicus* (Identified from IMTECH Chandigarh).

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

A total of twenty six morphologically different bacterial colonies were selected by crowded plate technique from different food samples and made pure culture. Each isolated strain was screened for antagonistic activity against standard bacterial cultures by paper disc method. Of those isolated bacteria only ISF-1, IN-1 and IPM-1 shown antimicrobial activity against standard bacterial cultures (MTCC 3041)(Pic-1).



Pic. 1: Anti-microbial activity of 14 hours grown culture of ISF-1, IN-1 and IPM against MTCC 3041 (*Lactococcus lactis subsp. lactis*).



Pic. 2: Morphological structure after 12 hrs. of growth (A) and after 18 hrs (B).

Morphological and biochemical characterization of ISF-1 was performed using 24h old cultures. Gram staining followed by microscopic observation showed that ISF-1 is gram positive rod shaped bacteria that exist in chains (pic-2).

From the result it was observed that ISF-1 and IN-1 were able to grow at 30°C, 37°C and 45°C but they grown maximally at 37°C and they are unable to grow at 4°C and 10°C. In case of pH tolerance ISF-1 and IN-1 were able to grow only at P^H-7 and unable to grow at P^H-3, P^H-5 and P^H-9. Isolates were also tested for their ability to use different sugars as their sole carbon source by growing the isolates in MRS broth containing different sugars (Maltose, Inositol, Trehalose, Dextrose, Galactose, Fructose, Cellobiose, Rhamnose, Lactose, Sucrose and Mannitol). It was found that ISF-1 was able to utilize all the sugars except cellobiose and IN-1 was able to utilize all the sugars except cellobiose and mannitol.

Fungi isolated from rotten vegetable/fruits like Mango (*Mangifera indica*), Lemon (*Citrus limon*), Kundri (Eng. Ivy guard) (*Coccinia grandis*), Pear (*Pyrus*), Bitter guard (*Momordica charantia*) and green potato (*Solanum tuberosum*) Among the three isolates IN-1 shown maximum anti-microbial activity against the fungi isolated from rotten fruits. Inhibition zone ranges between 1.8 – 2.5 cm for ISF-1, 2.4 – 3.6 cm for IPM-1, 2.1 – 2.7 cm for IN and 1.8 – 2.1 cm for *Lactobacillus*

plantarum (MTCC 3089, indicator organism) were observed (Table 1 and Pic-3).

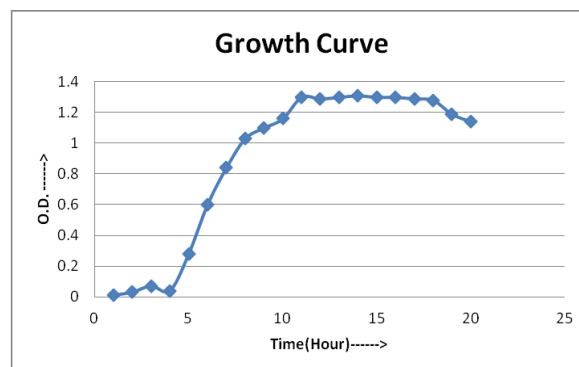


Figure 1: Growth curve of ISF-1.

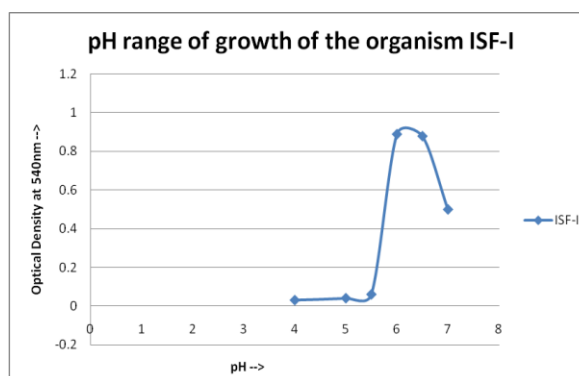
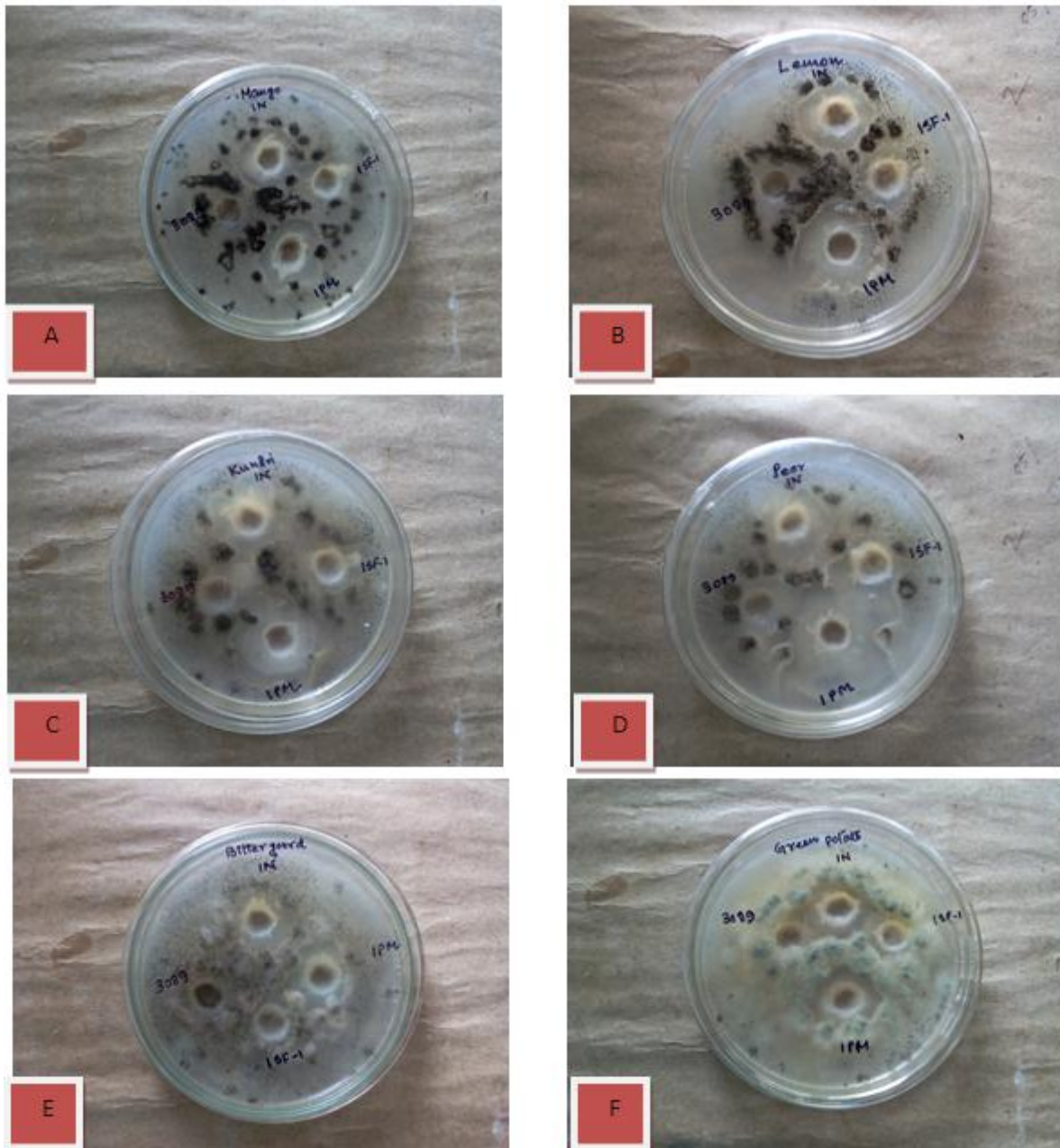


Fig. 2: Effect of growth on different pH.

Table 1: The clear zones surrounding the wells (the zone of inhibition) were observed and the results were recorded.

Serial No.	Source of spoilage fungi	Supernatant of the Isolates	Presence of zone of inhibition	Diameter of zone of inhibition in cm
A.	Mango	ISF-I	+	2.3
		IPM	+	2.7
		IN	+	2.6
		3089	+	2.0
B.	Lemon	ISF-I	+	2.1
		IPM	+	2.8
		IN	+	2.5
		3089	+	2.1
C.	Kundri	ISF-I	+	2.1
		IPM	+	3.0
		IN	+	2.6
		3089	+	1.8
D.	Pear	ISF-I	+	2.5
		IPM	+	3.6
		IN	+	2.7
		3089	+	2.1
E.	Bittergurd	ISF-I	+	2.0
		IPM	+	2.1
		IN	+	2.3
		3089	-	-
F.	Green potato	ISF-I	+	1.8
		IPM	+	2.4
		IN	+	2.1
		3089	-	1.8



Pic. 3: Inhibition zone observed in the plates containing different pathogenic fungus culture, A = Mango: B = Lemon: C = Kundri: D = Pear: E = Bitter Guard: F = Green Potato.

CONCLUSION

Microorganisms specially Lactic acid bacteria have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf-life characteristics. They cause rapid acidification of the raw material through the production of antimicrobial substances which causes the preservation of the nutritional qualities of the raw material through extended shelf life and the inhibition of spoilage and pathogenic bacteria. Several food-grade lactic acid bacteria, used in food fermentation, are known to have these antimicrobial properties. They provide safety and shelf-stability to the fermented foods.

In the present investigation it was observed that the isolate ISF, IPM and IN can inhibit the growth of the pathogenic fungi isolated from raw vegetables. The supernatant of isolate ISF-I, IN-1 and IPM-1 after 18 hours of growth produced clear zone of inhibition against spoilage fungi isolated from Kundri (Eng. Ivy guard) (*Coccinia grandis*), Pear (*Pyrus*), Bitter guard (*Momordica charantia*) and green potato (*Solanum tuberosum*) The range of inhibition zone diameter is between 1.8-3.6 cm. Morphological, physiological and biochemical characters of ISF-1 was studied and recorded. By 16S rRNA sequencing analysis isolate ISF-1 was identified as *Bacillus methylotropicus* (Identified from IMTECH Chandigarh). There is no such report of the antimicrobial activity of *Bacillus methylotropicus*.

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