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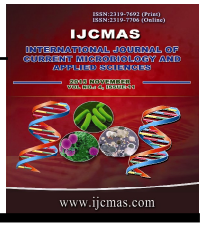

Original Research Article

Antimicrobial Attributes of *Leuconostoc* Isolates against Fruit and Vegetable Spoilage Organisms

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Hydatid cyst, Pancreatic hydatidosis

A B S T R A C T

In recent years, there has been considerable interest in the use of antagonistic microorganisms for the control of post-harvest diseases in fruits and vegetables. The present study is focused on the assessment of *Leuconostoc* spp. as biocontrol agents against fruit and vegetable spoilage organisms. *Leuconostoc* spp. were isolated from different fruits, vegetables, milk, milk products, pickles and sugarcane juice. They were subjected to morphological and biochemical characterization. These isolates were observed for their antimicrobial properties against the isolated spoilage organisms. The cell free extracts of six best isolates of *Leuconostoc* spp. were obtained and were tested for their antagonistic activity against the spoilage isolates. With reference to antibacterial activity against *Xanthomonas* sp. and *Erwinia* sp. and spoilage molds, *Leuconostoc* isolates could effectively inhibit bacteria than molds. Culture broth of LM 15 recorded the highest zone of inhibition (13.59 cm^2) followed by LM 30 (11.20 cm^2) against *Xanthomonas* sp. Culture broth was more effective than cell free extract. The pH of cell free extracts was neutralized to avoid the lactic acid effect, and then tested for activity of bacteriocin like substance (BCLS). The inhibition effect of BCLS activity was also more against bacteria than mold isolates.

Introduction

In recent decades, agriculture has undergone great changes to adapt itself to the fast evolution of the market and to the changing requirements of the consumer. Intensive agriculture has resulted in the development of particularly dedicated geographical areas, which has in turn created over production in specific zones and at certain times of the year. These products need to be subjected to varying types of transport and storage, in terms of the product characteristics and the market demands so as to distribute over time and space. The situation is more difficult to manage especially if fruits and vegetables are highly perishable. The request by consumers for fresh produce that is available throughout year and or comes from exotic production areas increases the need for storage and transport of fruits and vegetables.

The decrease in postharvest losses of fruits and vegetables can provide an effective way of increasing food availability and reducing the land needed for its production (Kader, 2005).

Generally post harvest spoilages are caused by wound pathogens. So, the research has been focused on those spoilage organisms and the biocontrol agents to check the spoilage. Biocontrol agents used in this study were *Leuconostoc* spp. The antimicrobial activity of *Leuconostoc* spp. against the spoilage microorganisms has been studied under *in vitro* conditions.

Material and Methods

Isolation of *Leuconostoc* spp.

Leuconostoc spp. was isolated using De Mann, Rogosa and Sharpe (MRS) medium. Healthy, fresh fruits and vegetable samples of grapes, papaya, mango, carrot, tomato, milk, curds and pickles were subjected to microbiological analysis for isolation of *Leuconostoc* spp. using standard plate count technique.

Isolation of spoilage organisms

Spoiled fruits and vegetables were collected from the local markets in sterile polythene bags. Infected tissue was removed using sterile knife and subjected for microbiological analysis, spoilage bacterial isolates were *Erwinia* spp. *Xanthomonas* spp. and fungal isolates *viz., Aspergillus* spp. *Penicillium* spp.

Test for infectivity of spoilage isolates

The spoilage isolates were tested for their infectivity by wound prick method. Healthy fresh fruits and vegetables were selected. Fruits were washed in tap water and soaked in 3 per cent solution of sodium hypochlorite for surface sterilization and known area of fruit and vegetables were marked on the surface and were bruised by pricking with sterile needles. The inoculum suspension of 48 h old cultures of spoilage bacteria and 72 h old cultures of mold suspensions were inoculated to the needle pricked area. The inoculated fruits were incubated in sterile polythene bags and observed for spoilage.

$$\text{Per cent spoilage} = \frac{\text{Number of samples spoiled}}{\text{Total numbers of sample}} \times 100$$

Antimicrobial activity of *Leuconostoc* isolates against spoilage bacteria and molds under *in vitro* conditions

Leuconostoc isolates were screened for their antimicrobial activity against spoilage microorganisms *viz., Aspergillus* sp., *Penicillium* sp., *Xanthomonas* sp. and *Erwinia* sp, isolated from spoiled fruits and vegetables. These organisms were chosen as they have been reported often as post harvest spoilage agents of fruits and vegetables and are known to cause considerable loss (Goland, 2001).

The diameter of clear zone around the well was measured. The area of inhibition was computed in terms of square centimeters. Higher area of zone of inhibition indicated greater antimicrobial activity. The area of zone of inhibition was calculated using the formula (Trias *et al.,* 2008).

Area of the zone of inhibition = π (R+r) (R-r)

Where R= is the radius of the zone of inhibition

r= is the radius of the agar well

Antimicrobial activity of cell free extracts against spoilage organisms

The cell free extracts of the efficient *Leuconostoc* isolates were used to study the antimicrobial activity by agar well diffusion assay technique (Casla *et al.,* 1996). Actively growing cultures of the selected *Leuconostoc* sp. were inoculated to MRS broth and incubated at room temperature for 36 h. Cells were removed by centrifugation at 12,000 rpm for 15min. The filtrates were used to evaluate antimicrobial activity by agar well diffusion method. Zone of inhibition around the wells depicted the antimicrobial activity. Comparative study between cell free extract and culture broth was carried out.

Effects of Bacteriocin like substances (BLSC) against the spoilage bacteria and molds

The selected *Leuconostoc* isolates were grown in MRS broth (pH 6.5) at room temperature. Broth was transferred aseptically to pre sterilized centrifuged tubes after it attained turbidity. The culture was centrifuged at 12,000 rpm for 15 minutes at 4 ^{0}C. The supernatant broth was loaded to pre sterilized syringe fitted with 0.45 µ membrane filter. The filtered cell free extract had acidic pH and it was adjusted to neutral pH by using 0.1 M NaOH to exclude the antimicrobial activity of organic acids (Schillinger *et al.,* 1991).

Actively growing cultures of *Erwinia* and *Xanthomonas* sp. having population of 10^5 cfu/ml were inoculated to molten nutrient agar medium at the rate of 2.5 per cent. Wells were scooped in the agar medium using sterile cork borer (7mm diameter) and cell free extract (50 µl) was inoculated into the well. The plates were incubated at room temperature for 36 h and observations were recorded.

Statistical analysis

The results of the experiments were analyzed using CRD and with Duncan's multiple range test for the test of significance by using M Stat software. The probability test was done with Turkey's test using systat 10.1 version soft ware.

Results and Discussion

Isolation of *Leuconostoc* spp. *Leuconostoc* spp. were isolated from different food sources such as from fruits vegetables, milk, pickles, sugarcane juice and green leaves. *Leuconostoc* spp. is present in many natural ecological niches like phyllosphere and roots from where they are easily propagated into various niches such as vegetable silage. Ennahars *et al.* (2003) reported that, they are commonly found in sugar processing liquors and fermented foods.

Isolation of spoilage organisms Spoilage bacteria were isolated from spoiled fruit and vegetable samples. Bhattacharya and Mukherjee (1986) found that *Erwinia* and *Xanthomonas* spp. were responsible for causing severe post harvest diseases of fruits and vegetables stored at normal temperature. Spadaro and Gullino (2004) reported that *Erwinia carotovora* and *Xanthomonas vesicatoria* have been described as common spoilage microorganisms of fresh fruits and vegetables. The dominant cultures, isolated from spoiled fruits and vegetables belonged to the genera of *Aspergillus* and *Penicillium* sp. These genera are the most commonly associated ones with fruits and vegetables. Samson (2001) reported that *Penicillium* sp. and *Aspergillus* sp. are responsible for causing spoilage in a wide range of fruits and vegetables.

Test for infectivity

The spoilage isolates of bacteria and molds

capable of hydrolyzing pectin were subjected to pathogenicity test by inoculating these organisms into healthy fruits and vegetables by pin prick method. Majority of the isolates expressed spoilage symptoms at different intervals.

Mold isolates APY and PLPM followed by X1a and ETT bacterial isolates caused severe infection. There are reports that *Aspergillus* sp. and *Penicillium* sp. cause post harvest diseases in fruits and vegetables (Akthar *et al.,* 2013), thus upholding our results.

Antibacterial activity of *Leuconostoc* isolates against isolated spoilage organisms

Leuconostoc isolates were tested against *Xanthomonas* and *Erwinia* isolates. It was seen that *Leuconostoc* isolates LM04, LM14, LM15, LM29 and LM30 exhibited the highest zone of inhibition compared to other isolates. *Leuconostoc* isolate LM04 showed the highest zone of inhibition against *Aspergillus* sp (APY) and *Penicillium* sp. (PPLM).

All other treatments were significantly differing from each other. These isolates were on par with LM(s) isolate. Klingberg *et al.* (2006) reported that *Leuconostoc* sp. inhibited the growth of several pathogenic organisms.

The inhibitory activity of *Leuconostoc* sp. could be attributed to the creation of hostile environment for pathogenic and spoilage organisms, even though, several mechanisms are elucidated for such effects, the net effect in terms of suppression of spoilage organisms is the result of more than one mechanisms operating against pathogenic and spoilage organisms.

Antimicrobial activity of cell free extracts of *Leuconostoc* isolates against spoilage organisms

Efficient six isolates were selected to study the effect of antimicrobial activity of cell free extracts of *Leuconostoc* isolates against the selected spoilage microorganisms. The highest inhibition was found with LM30 *i.e.* 2.51 cm^2. The culture broth was three times more effective than the cell free extracts. Cell free extracts three out of six could inhibit *Penicillium* sp. The highest inhibition of molds was recorded with LM04 cell free extracts followed by LM14 and LM30. The strains LM15, LM29 and LM(s) could not inhibit *Penicillium* sp. The inhibitory activity against molds was more in culture broth than cell free extracts. The culture broth was almost two times more effective than the cell free extracts. Similar types of results were also reported. Among both the mold species *Penicillium* sp. was the sensitive (Melin *et al.*, 2004).

Bacteriocin like substance activities (BLSC)

The pH of cell free extracts was neutralized to avoid the lactic acid effect, and then tested for activity of bacteriocin like substance (BLSC). LM 14 and LM30 showed the highest inhibitory activity against *Xanthomonas* sp. LM15 and LM29 were inactive against *Xanthomonas* sp. Similarly with *Erwinia* sp. LM15 and LM30 showed the highest inhibitory activity. LM30 showed the highest inhibitory effect against *Aspergillus* sp. LM30 and LM(s) showed the highest inhibitory activity against *Penicillium* sp. also. The inhibition effect of BLSC activity was more against bacteria than mold isolates.

Table.1 Antimicrobial activity of *Leuconostoc* isolates against spoilage isolates

***Leuconostoc* Isolates**	**Zone of inhibition (cm^2)**			
	***Xanthomonas* sp. (X1a)**	***Erwinia* sp. (ETT)**	***Aspergillus* sp. (APY)**	***Penicillium* sp.(PPLM)**
LM01	07.23^{abc}	00.00^{n}	4.06^{abc}	1.26^{ab}
LM02	09.65^{abc}	09.38^{abcde}	$2.20^{cd\ efg}$	1.91^{ab}
LM03	06.33^{bc}	09.34^{abcd}	3.82^{abcd}	1.13^{ab}
LM04	11.45^{ab}	12.51^{a}	5.25^{a}	3.32^{a}
LM05	09.18^{abc}	01.12^{lmn}	1.69^{efghi}	2.04^{ab}
LM06	05.20^{bcd}	11.36^{abcd}	3.46^{abcde}	2.01^{ab}
LM07	07.42^{abc}	06.42^{efghi}	2.01^{defgh}	1.59^{ab}
LM08	04.49^{cd}	05.78^{efghi}	3.31^{bcdef}	2.23^{ab}
LM09	07.41^{abc}	08.76^{bcdef}	1.85^{defghi}	1.91^{ab}
LM10	10.18^{abc}	09.04^{abcdef}	2.33^{cdefgh}	2.11^{ab}
LM11	08.54^{abc}	07.78^{defgh}	2.56^{cdefg}	2.46^{a}
LM12	07.41^{abc}	07.93^{cdefgi}	3.00^{bcdefg}	1.18^{ab}
LM13	09.34^{abc}	09.27^{abcdef}	2.66^{cdefg}	0.00^{b}
LM14	10.11^{abc}	11.59^{abc}	4.79^{ab}	3.24^{a}
LM15	13.59^{a}	12.22^{ab}	2.92^{bcdefg}	1.57^{ab}
LM16	03.29^{cd}	02.27^{klmn}	1.86^{defghi}	1.55^{ab}
LM17	07.28^{abc}	00.00^{n}	$2.11^{cdefghi}$	2.32^{ab}
LM18	00.00^{d}	04.39^{hijkl}	1.35^{fghi}	0.00^{b}
LM19	05.67^{bcd}	02.90^{jklmn}	2.99^{bcdefg}	2.52^{a}
LM20	08.59^{abc}	05.07^{ghijk}	1.63^{efghi}	2.53^{a}
LM21	10.65^{abc}	06.85^{efghi}	0.00^{i}	1.13^{ab}
LM22	07.03^{bc}	07.79^{defg}	1.68^{efghi}	1.60^{ab}
LM23	06.21^{bc}	06.08^{efghi}	0.00^{i}	1.28^{ab}
LM24	05.24^{bcd}	00.79^{mn}	2.04^{cdefgh}	0.00^{b}
LM25	08.89^{abc}	05.53^{fghijk}	0.96^{ghi}	2.07^{ab}
LM26	00.00^{d}	03.57^{ijklm}	2.37^{cdefgh}	2.27^{ab}
LM27	04.21^{cd}	00.00^{n}	$3.08b^{cdef}$	2.26^{ab}
LM28	08.33^{abc}	04.48^{hijk}	1.00^{ghi}	1.91^{ab}
LM29	11.26^{ab}	08.31^{cdefg}	1.49^{efghi}	2.56^{a}
LM30	11.62^{ab}	10.81^{abcd}	3.44^{abcde}	3.29^{a}
LM(S)	11.20^{ab}	08.87^{abcdef}	2.49^{cdefg}	1.62^{ab}

Table.2 Antimicrobial activity of cell free extracts of Leuconostoc isolates

***Leuconostoc* isolate**	**Zone of inhibition (cm^2)**							
	***Aspergillus* sp. (APY)**		***Penicillium* sp. (PPLM)**		***Xanthomonas* sp. (X1a)**		***Erwinia* sp. (ETT)**	
	Culture broth	Cell free extract	Culture broth	Cell free extract	Culture broth	Cell free extract	Culture broth	Cell free extract
LM04	5.25^{a}	2.14^{a}	3.32^{a}	3.21^{a}	11.45^{a}	4.93^{a}	11.36^{ab}	5.91^{b}
LM14	2.99^{ab}	0^{a}	2.32^{a}	2.51^{ab}	10.11^{a}	4.79^{a}	11.59^{ab}	6.14^{ab}
LM15	2.92^{ab}	1.31^{a}	1.57^{a}	0^{b}	13.59^{a}	5.57^{a}	12.22^{a}	7.37^{a}
LM29	1.49^{b}	0^{a}	2.56^{a}	0^{b}	11.26^{a}	4.73^{a}	$8.31b^{c}$	4.92^{bc}
LM30	3.44^{ab}	2.51^{a}	3.29^{a}	2.26^{ab}	11.62^{a}	4.84^{a}	7.78^{c}	4.42^{c}
LM(S)	2.49^{b}	2.05^{a}	1.62^{a}	0^{b}	11.20^{a}	5.98^{a}	8.87^{abc}	4.35^{c}
MEANS	3.09	1.33	2.44	1.33	11.53	5.14	10.02	5.51

Note: Values are average of three replications.

The present study leads to a conclusion that *Leuconostoc* spp. have a major potential use in biopreservation because they are safe to consume and during storage they naturally dominate the microflora of many foods. They have Generally Recognized As Safe (GRAS) status (Stiles, 1996).

The ability of *Leuconostoc* spp. to promote safety or quality is linked to excreted organic acids subsequent pH reduction and many other antimicrobial compounds such as CO_2, diacetyl, hydrogen peroxide and bacteriocins (Hemme and Foucaud, 2004). Bio preservation is gaining popularity as it forms a part of organic farming.

There is an increasing passion for eco friendly products by the public. Commercial production of dextrans and levans by *Leuconostoc mesenteroides* for use in biochemical and pharmaceutical industry has been carried out for more than 50 years. Dextrans are used in the manufacture of blood plasma extenders, heparin substitutes for anticoagulant therapy, cosmetics and other products (Sutherland, 1996).

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