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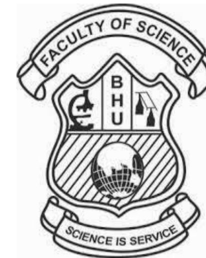

Antifungal Activity of Plant Isolate of Lactic Acid Bacteria against *Fusarium Sp.*

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Abstract: The lactic acid bacteria is one of the most diverse groups of bacteria, and these organisms have been characterized extensively by using different techniques. Lactic acid bacteria are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins. These are considered as 'Generally Recognized as Safe' (GRAS) organisms. LAB have been shown to occur in fruits, flowers and vegetable matrices.60 isolates from plant sources were screened for antifungal activity. The isolate showing maximum antifungal activity against *Fusarium sp.* was identified using 16s rDNA technique to be *Leuconostoc mesenteroides* strain and studied for production of different antifungal compounds. The results indicated that the *Leuconostoc mesenteroides* from plant source produces a wide variety of antifungal compounds and is suggestive of applications for preservation of agriculture produce.

Index Terms: Antifungal compounds, Characterization, Inhibition, Lactic acid bacteria, Plant sources.

I. INTRODUCTION

The presence of lactic acid bacteria (LAB) in dairy products has long been established but the occurrence of this particular group of bacteria on fruits and vegetables has rarely been focused. (Naeem et al, 2012**)**. LAB are naturally present on most living plants (Etchells et al, 1961; Mundt, 1970). Fresh vegetables constitute a good source of lactic acid bacteria with the ability to inhibit wide range of spoilage fungi (Sathe et al, 2007). LAB have been isolated from fresh vegetables, carrots, lettuce, cucumbers, parsley and cabbage (Adeniyi et al, 2011). LAB have been shown to occur in fruits, flowers and vegetable matrices (Annacarso et al ,2015). Mulberry fruits harbor Lactic acid bacteria (Chen et al,2010).Rhizosphere of olive trees has been reported to be a good habitat of different species of LAB (Fboula et al ,2013). LAB have been isolated from golden apples and Iceberg lettuce (Trias.et al, 2008; Matei et al, 2014). Magnusson et al, (2003) have isolated LAB from various environmental sources.

Presence and isolation of antifungal LAB has been reported by many researchers. Though dairy fermented products remain a favourite in these findings, antifungal isolates from plant sources are reported. Trias et al (2008) have reported antifungal LAB from various fresh fruits and vegetables, processed and unprocessed. Isolatiom of antifungal LAB from grass, wheat, corn, maize, barley and alfa alfa silage has been reported by many workers. (Kung & Ranjit, 2001; Driehuis et al, 2002; Taylor & Kung, 2002; Ranjit et al, 2002; Nishino et al, 2004; Zhang et al , 2009).

Fungal spoilage of fresh fruits and vegetables is a main cause of concern for post-harvest technology. The most important microorganisms involved in deterioration of agricultural produce are fungi. Agricultural produce which are stored with excessively high moisture content will become heavily infected by molds and fungal infection can very easily result in complete destruction of the agricultural produce. Several species of fungi which may infect agricultural produce are capable of producing mycotoxins. These are extremely toxic compounds, the presence of which renders agricultural produce unsuitable for human or animal consumption.

A continuous demand for reduction of pesticide content in our food and the environment has caused a concern over our present control practices for postharvest diseases. The challenge and opportunity to develop safe and effective alternatives to present-day synthetic fungicides is gaining pace. The climate for support of biological control research is now excellent.

The use of lactic acid bacteria to combat fungal decay of agricultural produce is a promising solution. The study and application of antifungal LAB has evoked a considerable interest in recent years. Significant progress has been reported

on the isolation and characterization of antimycotic compounds. The "Generally Recognized as Safe" (GRAS) status of LAB, offers the potential to use these bacteria as biological control agents in post harvest produce to prevent fungal growth and reduce the health hazards associated with mycotoxins.

Hydrogen peroxide, carbon dioxide, aromatic compounds like diacetyl, organic acids like lactic acid, acetic acid, and propionic acid are shown to have antifungal activity. Phenyl lactic acid (PLA) is perhaps the most elaborately studied antifungal organic acids from LAB. (Dal Bello et al, 2007; Rizzello et al,2011; Ryan et al, 2011; Strom et al, 2002). Fatty acids produced by LAB are known to show antifungal activity. (Bergsson et al , 2001; Thormar,2001).Reuterin,a low molecular weight compound has been shown to have antifungal effect. (Nakanishi et al, 2002). Antifungal activities have been identified for cyclic dipeptides (Strom et al, 2002) and various proteinaceous compounds (Coda et al, 2008; Rizzello et al, 2011) produced by LAB. Apart from these major antifungal compounds a wide range of various miscellaneous compounds have been reported which show inhibitory activity against different types of plant pathogenic fungi.

The current knowledge of antifungal LAB, their bioactive metabolites, applications in post harvest preservation systems and interactions with their target fungi will provide a safer alternative to overcome the post harvest losses.

II.MATERIALS AND METHODS

A. Isolation and Characterization

Fifty six isolates of LAB were obtained from Department of Microbiology Savitribai Phule Pune University,Pune,Maharashtra,India. The isolates were from aerial parts of the plants like leaves, buds, flowers and fruits. The isolates were subcultured on MRS medium (Hi-Media). Regular transfers were given to maintain the isolates.

Gram staining was performed for all the isolates. Crystal violet was used as primary stain and safranin was used as counter stain. Acetone alcohol mixture was used as decolorizer.

To check motile nature of the isolates hanging drop preparation was done for each of the isolates and nature of motility was noted for all the isolates. (Collins, 2004)

B. Nature of Fermentation

To check whether the cultures are homofermentative or heterofermentative, Gibbsons tomato juice medium (Hi Media) was used. It contains tomato juice, skimmed milk powder, and nutrient agar with yeast extract. Each of the isolates was streaked on this medium. Slants were incubated for 48 hours under microaerophilc conditions. Presence of gas bubbles trapped in the medium or uplifting of the medium were observed. Presence of gas bubbles indicates heterofermentative nature while their absence marks homofermentative nature.

C. Biochemical Tests

The ability of the isolates to produce catalase was checked. This was done by streaking the cultures on MRS medium (Hi Media) and then incubating for 48 hours under microaerophilic conditions.H2O2(3%)was poured on the slants and observation was made for evolution of oxygen bubbles.

D. Genotypic Identification

The isolate was grown in MRS broth under microaerophilic conditions for 48 hours.The suspensions were centrifuged for 25 minutes at 10,000 rpm. Supernatent was discarded and cell pellet was collected. This cell pellet was processed for DNA isolation according to the protocol (Ausubel FM et al, 1987).

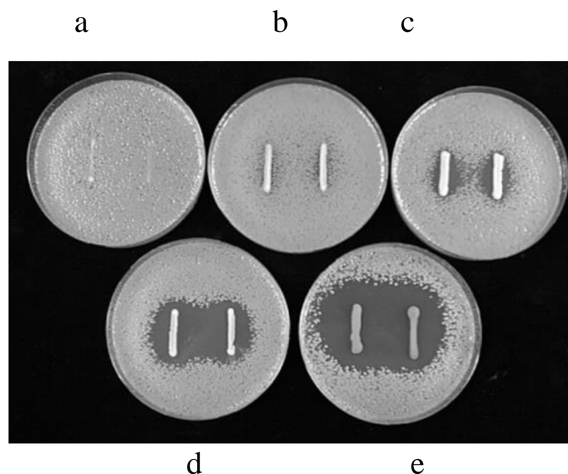
The isolated DNA was sent to NCCS, Pune, India for identification. Genus level identification was carried out using 16S rDNA technique.

E. Checking of Antifungal Activity

All the LAB isolates were screened for the production of antifungal compounds. The phytopathogenic fungus *Fusarium oxysporum* was used as target fungus.

The LAB isolates were screened for antifungal activity in a dual culture assay on MRS agar plates. LAB isolates were grown in two lines on the plate under microaerophilic conditions in a dessicator. After incubation, the plate was overlaid with Czapek Dox soft agar (1% agar) containing fungal spores (106 spores/ml). If clear zones of inhibition were present on the plates (Fig: Katrin Strom, Doctoral Thesis, 2005) isolates were considered to possess antifungal activity and selected for further evaluation and characterization.

a b c



d e

a. No Inhibition, b. (+) weak inhibition, c. + Inhibition, d. ++ Inhibition e. +++Inhibition

Fig. 1 Gradation of antifungal activity

The antifungal activity of the isolates was graded according to the Fig 1 and the selection of LAB isolates showing good fungal inhibitory activity was made.

F. Characterization of LAB Showing Antifungal Property

Twenty-six isolates showed antifungal activity and hence were chosen for further characterization. Out of the 26 LAB isolates which showed antifungal activity when tested against the phytopathogenic fungus *Fusarium oxysporum*, selection of the isolates having a broad spectrum of antifungal activity was made after testing their ability to inhibit a variety of phytopathogenic fungi. This was done against the fungal isolates obtained from National Research Centre on Pomegranate (NRC) Solapur, Maharashtra,India.
The ability to inhibit the fungi was checked by Agar overlay method as described above. Each of the 26 LAB isolates was checked against eight different fungi. Depending upon the ability to inhibit the growth of maximum number of fungi selection of the best isolates was made.
The selection of the best producer was done on the basis of the antifungal ability of the isolates against different fungal plant pathogens of pomegranate obtained from National Research Centre on Pomegranate,Solapur,Maharashtra ,India. On observing the results five isolates were selected for further studies on antifungal activity. These isolates were identified using 16SrDNA technique. The DNA sequences were submitted to the Gene Bank and accession numbers obtained.

FL3 (3) *Leuconostoc mesenteroides KM496471*
PL2 (3) *Leuconostoc mesenteroides* KM496472
PFL4 *Leuconostoc mesenteroides* KM496473
F3415 *Leuconostoc mesenteroides* KM496474
FL2 (5) *Lactobacillus brevis KM496475*

G. Checking of antifungal compounds.

1) Production of Crude Antifungal Preparation

The growth of LAB was suspended in saline to a final turbidity of 1OD (equivalent to 109cells /ml). It was inoculated in 500 ml of sterile MRS broth to a concentration of 106 cells /ml. The flask was incubated statically for 48 hours at 280C.The culture supernatant was prepared by centrifugation at 10,000 rpm for 10 mins followed by filtration through 20 µm syringe filter. (Axiva)

2) Slope Ratio Assay for Determining Potency of Antifungal Preparation

For determining the potency of the antifungal compound produced, Amphotericin B was used as the standard antibiotic. Serial dilutions of Amphotericin B were made in sterile phosphate buffer (pH 6.8) as directed. Similarly serial dilutions of culture supernatant of each of the isolates were made. The inhibition assay for the dilutions of Amphotericin B and CS of each isolate was done using standard disc diffusion assay method (Rex et al ,2001) by using *Fusarium oxysporum* (108 cfu/ml) as test fungus on Czapek Dox agar (Hi Media).
The plates were incubated at 28^0C for 48 hours to observe the zones of inhibition shown by Amphotericin B as well as by the CS of all the isolates. The inhibition zones were measured and observations made. These observations were subjected to the standard software for calculation of potency of the drug with slope ratio assay method. The potencies of CS from each of the isolates was calculated. Similar method was used by taking Benzamidizole and Nystatin as standard antibiotics.

3) Characterization of Antifungal Preparation

a) Activity of Culture Supernatant at Different pH

To determine the effect of varying pH on antifungal activity, the CS was distributed in 5ml aliquots. The pH of aliquots was adjusted using 1N HCl and 1N NaOH to 3, 4, 5, 6, 7,8 and 9. The aliquots were filtered through 20 µm syringe filter and their activity was determined using disc diffusion assay method. Uninoculated sterile MRS broth was adjusted to these pH values and used as control.

b) Activity of Culture Supernatant at Different Temperatures

To determine the effect of varying temperatures on antifungal activity, the CS was distributed in 5ml aliquots. The aliquots were exposed to 450C,500C,600C,800C and 1000C in water bath for 1 hour and in autoclave at 1210C for 15 mins. CS were cooled to room temperature and then antifungal activity determined by disc diffusion assay. Uninoculated sterile MRS broth exposed to above temperatures served as control.

c) Activity of Culture Supernatant on Treatment with Proteolytic Enzymes

To determine the effect of proteolytic enzymes on antifungal activity, CS was distributed in 10ml aliquots. The pH was adjusted to 7.6 for treatment with proteinase k (Sigma) and pepsin (Sigma) (Magnusson and Schnurer, 2001).The enzymes were added separately to a final concentration of 1mg /ml. The mixture was incubated in water bath at 370C for 1 hour. After incubation the pH was readjusted to initial pH of 4.5 and antifungal activity was determined by disc diffusion assay. Uninoculated sterile MRS broth with proteolytic enzymes served as control.

d) Activity of Culture Supernatant on Treatment with Lipolytic Enzymes

To determine the effect of lipolytic enzymes on antifungal activity, CS was distributed in 10ml aliquots. The pH was adjusted to 7.6 for treatment with lipase (Sigma) (Magnusson and Schnurer 2001).The enzyme was added separately to a final concentration of 1mg /ml. The mixture was incubated in water bath at 370C for 1 hour .After incubation, the pH was readjusted to initial pH of 4.5 and antifungal activity was determined by disc diffusion assay.

4) Comparison of Production of Antifungal Preparation Under Shake Flask and Stationary Conditions

Each of the LAB isolates was inoculated in sterile MRS broth (100ml at pH 6.5).One set was incubated at 300C for 48 hours on shaker and other set was incubated at 300C for 48 hours under stationary conditions. The culture supernatant was prepared by centrifugation at 10,000 rpm for 10 mins followed by filtration through 20 µm syringe filter. (Axiva make).The antifungal activity was determined by disc diffusion assay for both the conditions.

5) Optimization of Production Parameters

a) pH

Each of the LAB isolates was streaked separately on overnight dried sterile MRS agar plates of different pH(4.5,5.5,6.5,7.5,8.5 and 9) and incubated at 300C for 48 hours. The antifungal activity was determined by agar overlay technique as described earlier.

b) Temperature

Each of the LAB isolates was streaked separately on overnight dried sterile MRS agar plates of pH 6.5 and incubated at different temperatures (100C, 300C and 450C) for 48 hours. The antifungal activity was determined by agar overlay technique as described earlier.

c) Sugars

Each of the LAB isolates was streaked separately on sterile MRS agar plates in which dextrose was replaced with fructose,lactose,sucrose,maltose and mannitol.and incubated at 300C for 48 hours. The antifungal activity was determined by agar overlay technique as described under 2.4.

d) Inoculum Size

Sterile MRS broth of pH 6.5 was dispensed in 400ml quantity in 500ml Erlenmeyer flask and LAB (104cfu/ml) were inoculated at 0.1,0.2,0.3 0.4 and 0.5 %(v/v).The flasks were incubated at 300C for 48 hours. CS was prepared and antifungal activity checked by disc diffusion assay.

e) Volume of Medium to Space Ratio

LAB (104cfu/ml) was inoculated at 0.2 %(v/v) into 500ml flasks containing varying volumes of MRS broth viz. 00, 150, 200, 250, 300, 350, and 400 ml. The flasks were incubated at 300C for 48 hours.CS was prepared and antifungal activity checked by disc diffusion assay.

f) Time Course

LAB (104cfu/ml) was inoculated at 0.2%(v/v) into 500ml flasks containing 400ml of MRS broth. (Sathe et al, 2007). Aliquots were withdrawn every 2 hours and CS prepared. Antifungal activity checked by disc diffusion assay. Assay was done in triplicates. Other observations like growth in terms of cell density (OD at 600nm) and pH of CS were also made.

6) Growth and Production of Antifungal Compounds in Different Media

a)Ability to Grow on Different Media

To check the growth of antagonistic LAB on different media, LAB cell suspension was streaked on sterile Ellikers Agar,Tryptose Soya Agar,TYD Agar,APT agar,Malt Extract agar, Pomegranate Juice Agar, Rind Agar, Corn Meal agar, MRS agar of pH 6.5.The plates were incubated at 300C for 48 hours and growth of each LAB isolate was checked on each of the media.

b) Ability to Produce Antifungal Compounds on Different Media

To check the production of antifungal compounds by antagonistic LAB on different media LAB cell suspension was streaked on sterile Ellikers Agar, Tryptose Soya Agar, TYD Agar, APT agar, Malt Extract agar, Pomegranate Juice Agar, Rind Agar, Corn Meal agar, MRS agar of pH 6.5. The plates were incubated at 300C for 48 hours and growth of each LAB isolate was checked on each of the medium. The media on which LAB showed growth were chosen for demonstration of antifungal activity which was checked by agar overlay method as described earlier.

c) Comparison of Inhibition Activity on Different Media

The % inhibition in all the above mentioned media was done by performing the Dual culture Technique in liquid media. This was done by inoculating LAB (104cfu/ml) in 10 ml aliquots of each of the media separately. The tubes were incubated at300C for 48 hours. The test fungus 10µl of 108 cfu/ml was inoculated in each tube with LAB growth. The tubes were again incubated at300C for 48 hours. After incubation the growth from each tube was filtered through Whatman Filter paper, dried and weighed. Controls were maintained for LAB and for test fungus. This was done for all the above media. The antifungal activity was calculated using the formula:

$$\frac{\text{Control} - (\text{LAB} + \text{Fungus}) \times 100}{\text{Control}}$$

d) Selection of Best Medium

Based on the results obtained from dual culture technique the data was analysed appropriately by calculating Standard Deviation, performing ANOVA and calculating p values, the best medium for production of antifungal compounds was selected

7) Extraction of Antifungal Compounds

a) Antifungal Activity of Different Solvent Extracts

To extract the antifungal compounds in solvents butanol, hexane, chloroform and ethyl acetate were used as the solvents. The CS was prepared for each isolate grown in MRS broth and extracted with different solvents. For solvent extraction the following protocol was followed.

Culture supernatant + Solvent -----------→1:1 proportion

↓

Shake for one hour to mix

↓

Remove aqueous layer by using separating funnel

↓

Centrifuge the organic layer at 5000rpm for 15 mins

↓

Add anhydrous Na_2SO_4

↓

Leave overnight to evaporate the solvent

↓

Dissolve the residue in Distilled Water and evaporate in oven (45°C)

↓

Redissolve in double Distilled Water

The solvent extracts were checked for antifungal activity using disc diffusion assay.

b) Silica Gel Column Chromatography

The ethyl acetate extracts for all the LAB isolates were subjected to Silica gel column chromatography to obtain fractions. Pure silica was completely dried in hot air oven before packing in column. A clean dried glass column was used to pack silica. A column of 10cm was prepared using HPLC grade methanol. Two ethyl acetate washes were given to the column.

Extracted compound (0.1mg) was dissolved in 1.5 ml of ethyl acetate and loaded on top of the column. The mother solvent used for eluting the fractions was used in 5 different combinations as follows.

100% ethyl acetate
75% ethyl acetate and 25% methanol
50%ethyl acetate and 50% methanol
25%ethyl acetate and 75% methanol
100% ethyl acetate
Each of the solvent combination (50 ml) was used. Fractions were collected as 3ml aliquots by maintaining a flow rate of 60 ml per hour i.e 1ml per min.
Collected fractions were completely dried in hot air oven (45°C)

c) Antifungal Activity of Collected Fractions

The dried fractions were resuspended in 10µl of ethyl acetate and antifungal activity was checked for each of these fractions obtained from each of the 5 LAB isolates by performing disc diffusion assay on Czapek Dox agar (Hi Media).Ethyl acetate was used as control. The plates were incubated at 300C for 48 hours and observed for zone of inhibition.

d) GC-MS Analysis of Collected Fractions

The fractions which demonstrated an antifungal activity greater than that of ethyl acetate were collected and sent for GC-MS analysis. A GCD-HP1800A GC/MS instrument (Helwett-Packard make USA) was used. The temperature of GC oven

was initially kept at 1200C and then increased to 2800C at 400C per 3 mins. Helium was used as carrier gas at a flow rate of 30ml /min. The identification of compounds was based on the similarity between MS spectra of the unknown and the reference compounds present in MS spectra library.

III.RESULTS AND DISCUSSIONS

The phenotypic observations were done based on morphology, as LAB are Gram positive rods or cocci which are non-motile. LAB are catalase negative which was confirmed by performing the test. The isolates which satisfied these criteria of being Gram positive, non-motile, rods or cocci and did not produce catalase were primarily selected as LAB.

All the isolates showed heterofermentative mode of fermentation when checked in Gibbsons Tomato juice medium Heterofermentative lactic acid bacteria such as Weissella and Leuconostoc and some lactobacilli produce equimolar amounts of lactate, CO_2 and ethanol from glucose via the hexose monophosphate or pentose pathway (De Vuyst et al, 1993; Axelsson et al, 1998).

On the basis of the results obtained for 16SrDNA identification the isolate belonged to genus *Leuconostoc*. Very little attention has been given to LAB which are present in abundance on plant material. These LAB also possess great potentials as their characters and biochemical nature is similar to of those from other classically exploited environments. They should be explored further for various probiotic nature, antifungal nature and PGPR traits. Out of the five isolates which were identified, PL2 (3), *Leuconostoc mesenteroides* KM496472 was used for further studies.

The isolate showed growth under aerobic and microaerophilic conditions.

The isolate showed homogenous growth in MRS broth as against most of the LAB cultures.

The isolate showed EPS production in EPS production medium.

The isolate showed oxidative fermentative nature in HL medium.

The isolate failed to grow at 10^0C but grew well at 45^0C.

The isolate grows well at pH 4.4 as well as at pH 9.6.

The isolate grows well at NaCl concentration of 0.5% and 5% but not at 8% and above.

The isolate showed acid production when grown in galactose, sucrose, maltose and mannitol.

The isolate showed growth on amylase production medium but did not produce amylase, it could not grow on cellulase and chitinase production medium but showed production of protease.

The isolate showed utilization of amino acid leucine for growth but did not utilize histidine, phenylalanine, alanine, glutamic acid and aspartic acid.

The isolate showed luxuriant growth in pomegranate juice at pH 6.5 but not at pH 3.5 under aerobic growth conditions as compared to microaerophilic conditions for growth.

The potency as determined by the slope ratio assay for the isolate was 0.9.

The isolate showed maximum antifungal activity at pH3 and pH4 where as minimum activity was observed at pH8 and pH9. (Table I)

The isolate showed maximum activity at 30^0C where as lower and higher temperatures showed decrease in inhibition activity. (Table I)

Table I: Antifungal activity of culture supernatant at varied values of parameters

parameters	Value/unit/type	Inhibition Zone Diameter (mm) by LAB
		Leu mes
		PL2(3)
pH	4.5	18
	5.5	23
	6.5	23
	7.5	20
	8.5	11
Temperature (°C)	10	12
	30	23
	45	ND
Sugar	Glucose	23
	Fructose	19
	Maltose	21
	Lactose	19
	Sucrose	21
	Mannitol	20
Inoculum Size (%)	0.1	11
	0.2	23
	0.3	21
	0.4	20
	0.5	19
Volume of medium /space (%)	20	14
	30	18
	40	18
	50	19
	60	20
	70	22

	80	23

ND: Not Detected

Treatment with both the proteolytic enzymes resulted in loss of antifungal activity indicating participation of proteinic compounds in antifungal activity.Treatment with lipases was without any effect on antifungal activity.

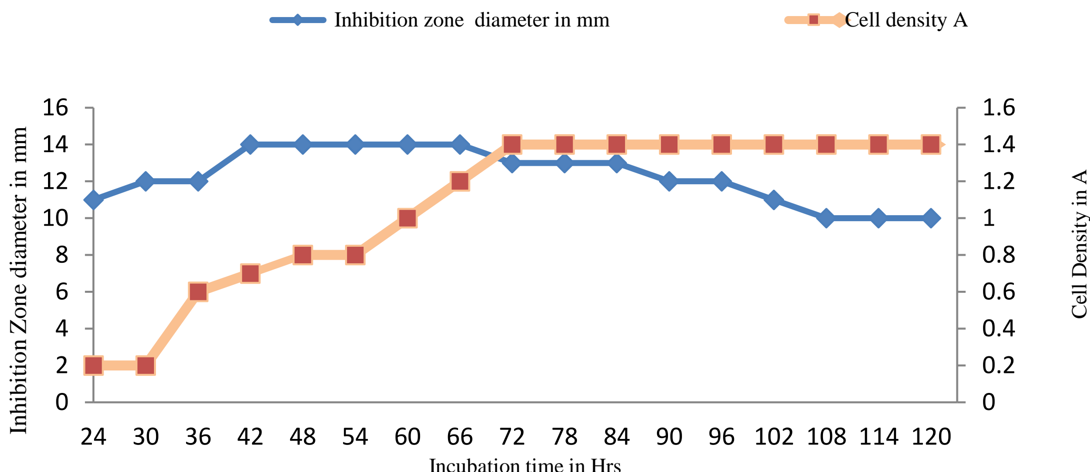


Fig.2 Time course of antifungal production by *Leuconostoc mesenteroides* PL2 (3)

The comparison of antifungal activity when production was carried out under stationary and shake flask conditions showed the stationary growth conditions to be favourable for production (Table II). The production was drastically decreased under shake flask condition. (Table III)

Table II: Production of antifungal compounds by LAB under stationary conditions

	Inhibition zone diameters in mm						
	Samples			Average	Rounded	SD	% Activity
LAB Isolates	1	2	3				
*Leu.mes*PL2(3)	23	22	23	22.67	23	0.47	90.67

Table III: Production of antifungal compounds by LAB under submerged conditions

	Inhibition zone diameters in mm						
	Samples			Average	Rounded	SD	% Activity
LAB Isolates	1	2	3				
*Leu.mes*PL2(3)	12	14	14	13.33	14	0.94	53.33

Table IV: Antifungal activity of Culture Supernatant (CS) of LAB grown in different media

	% Inhibition of *Fusarium oxysporum* by CS of LAB grown in									
LAB	PJM	MEM	RM	EB	TSB	YGB	TYGB	APTB	CCB	MRS
Leu mes	0	20	16	0	0	0	0	0	0	30
PL2(3)	0	20	17.2	0	0	0	0	0	0	30
	0	19	15.7	0	0	0	0	0	0	31.2
SD P	**0**	**0.471**	**0.648**	**0**	**0**	**0**	**0**	**0**	**0**	**0.566**

PJM : Pomegranate Juice Medium , MEM : Malt extract Medium ,RM : Rind Medium, EB :Ellikers Broth, TSB : Trypticase Soya Broth, YGB :Yeast Glucose Broth, CCB : Corn Crush Broth, MRS : DeMann Rogosa Sharpe medium.

Though there are numerous reports on use of different media for the growth of LAB scarce reports are available on comparison of media for the maximum production of antifungal compounds from LAB. This was done in the present study. On the basis of results obtained for antifungal production (Table IV) it was observed that MRS medium, Malt extract medium and Pomegranate Rind medium showed the best ability to help production of antifungal compounds. Rind medium was used as the isolate was from Pomegranate plants. Though rind medium shows good antifungal production, also being formulated from waste of Pomegranate could have proved to be a good alternative for growth and antifungal production but the rind in itself contains a few polyphenols and acidic contents which vary with the variety of fruits (Akbarpour,2010). Hence this medium was not used in further studies. On comparing the inhibition data for MRS and Malt extract medium, MRS showed better antifungal production and hence was used for further studies.

MRS medium was found to be the best medium for growth and production of the antifungal compounds. Hence on setting the various parameters like pH, temperature, type of sugar, inoculum size and the space to volume ratio, the optimization studies were carried out for various media components.MRS medium was prepared to check the growth of isolates and antifungal activity associated deleting one component from the medium each time. This was carried out in triplicates. It was observed that when glucose was deleted from the medium there was no growth of LAB isolates where as deletion of any of other media components didn't show any effect on growth. This reflects on glucose being the most essential carbon source in the medium and its inclusion in the medium since its absence cannot be compensated by any other medium component.

Absence of all other components one at a time from the medium didn't show an effect on growth indicating the fulfilment of the requirement of one component by the presence of other. (Hayek& Ibrahim, 2013). As observed for growth of LAB, the antifungal property too was not hampered when one factor was deleted at a time.

In case of the salts, it was observed that absence of all salts from the medium though resulted in growth of the isolates it failed to produce the antifungal activity. Deleting one metal salt at a time didn't have an effect on growth or antifungal activity. This shows the importance of salts for production of antifungal compounds and revealing the fact that metals can replace each other, some metals adsorb others, some metals interact differently in the presence of others. In addition, when omitting individual metals to determine their essential role for growth it is possible that small contaminations from other medium compounds or from the glassware may lead to faulty results. In spite of this, the role of metals in production of antifungal compounds cannot be overlooked.

Table V: Antifungal activity of CS of PL2 (3), *Leuconostoc mesenteroides* produced at different levels of micronutrients

Experiment no.	Inhibition zone diameter in mm		
	Set 1	Set2	Set3
1	-	-	-
2	-	-	-
3	-	-	-
4	12	12	12
5	12	12	12
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	11	11	11
13	16	16	16
14	-	-	-
15	-	-	-
16	12	12	12

Results of the **13th** experiment show increased antifungal activity (Table V). The potency of antifungal compounds produced by the LAB isolate PL2 (3), *Leuconostoc mesenteroides* was seen to increase by **65%**

The most suitable proportions of various salts were observed as follows:

Ammonium Citrate 0.4mg/100ml
Sodium acetate -0.1mg/100ml
Magnesium sulphate-0.02mg/100ml
Manganese sulphate-0.005mg/100ml
Dipotassium phosphate -0.2mg/100ml

The results show that a combination of the metal salts in the medium where proportion of Ammonium Citrate is doubled, sodium acetate is halved, Magnesium sulphate is doubled, and concentrations of Manganese sulphate and Dipotassium phosphate remain unchanged.

Since LAB are not naturally optimized for maximal production rates of biotechnologically important compounds, it is important to optimize, select, stabilize, and/or enhance the metabolic processes with regard to desired end products (Hayek& Ibrahim, 2013).

Such media optimisation studies have not been reported for antifungal production by LAB though there are few reports of such studies for bacteriocin production (Onwuakor et al, 2014). However, only a few nutritional parameters can be controlled at a time while holding other parameters constant. In addition, the fastidious characteristic of LAB, the ability of LAB strains to produce acid and antimicrobial compounds, and the variations in nutritional requirements among LAB strains may introduce additional limitations and challenges. Forming a culture medium that can deal with all or even most of the parameters and limitations continues to be a challenge. Accordingly, chemically defined media were suggested in many studies as an alternative to deal with different limitations and challenges. Chemically defined media remain important to study LAB, when it is necessary to consider all possible substrates and control available energy, carbon, and nitrogen sources that might influence growth and metabolism.

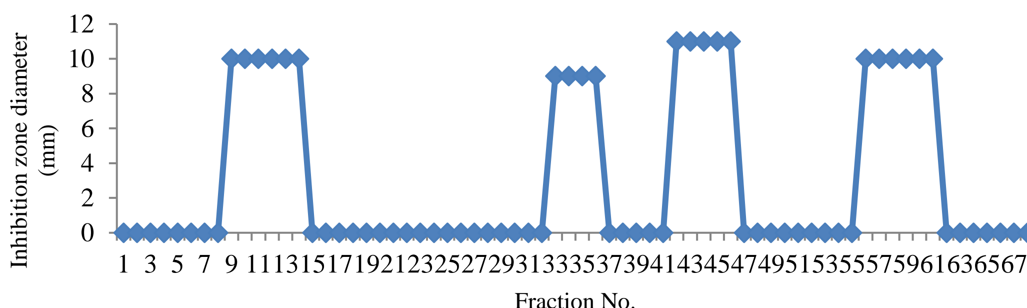
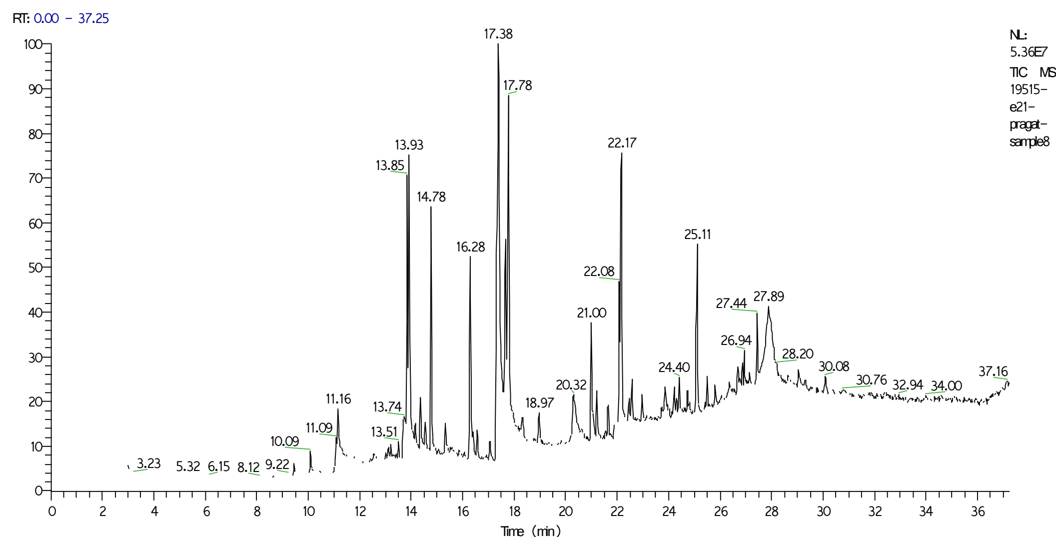


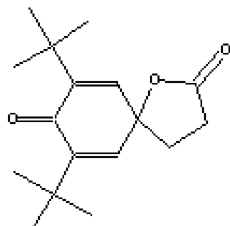
Fig.3 Active fractions collected after silica gel column chromatography of Ethyl acetate extract of CS of *Leuconostoc mesenteroides* PL2(3) against *Fusarium oxysporum*



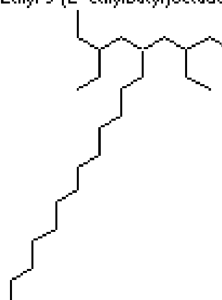
1-Nonadecane
Formula $C_{19}H_{38}$, MW 266, CAS# 18435-45-5, Entry# 791

Octadecane
Formula $C_{18}H_{38}$, MW 254, CAS# 593-45-3, Entry# 5445
n-Octadecane

7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
Formula $C_{17}H_{24}O_3$, MW 276, CAS# 82304-66-3, Entry# 5238
1-Oxa-spiro[4.5]deca-6,9-diene-2,8-dione, 7,9-di-tert-butyl-

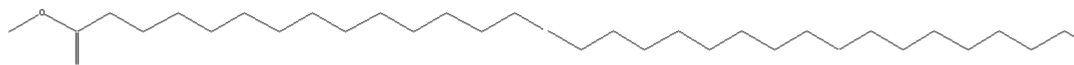


Octadecane, 3-ethyl-5-(2-ethylbutyl)-
Formula $C_{26}H_{54}$, MW 366, CAS# 55282-12-7, Entry# 2126
3-Ethyl-5-(2'-ethylbutyl)octadecane

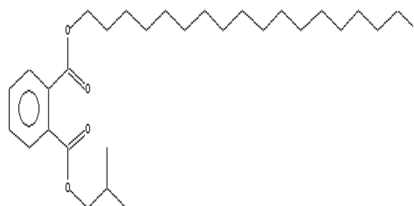


Heptadecanoic acid, 16-methyl-, methyl ester
Formula $C_{19}H_{38}O_2$, MW 298, CAS# 5129-61-3, Entry# 35281
Methyl isostearate

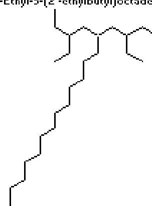
Eicosane
Formula $C_{20}H_{42}$, MW 282, CAS# 112-95-8, Entry# 5706
n-Eicosane



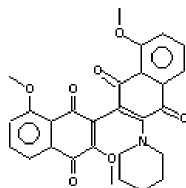
Phthalic acid, isobutyl octadecyl ester
Formula $C_{30}H_{50}O_4$, MW 474, CAS# NA, Entry# 35333

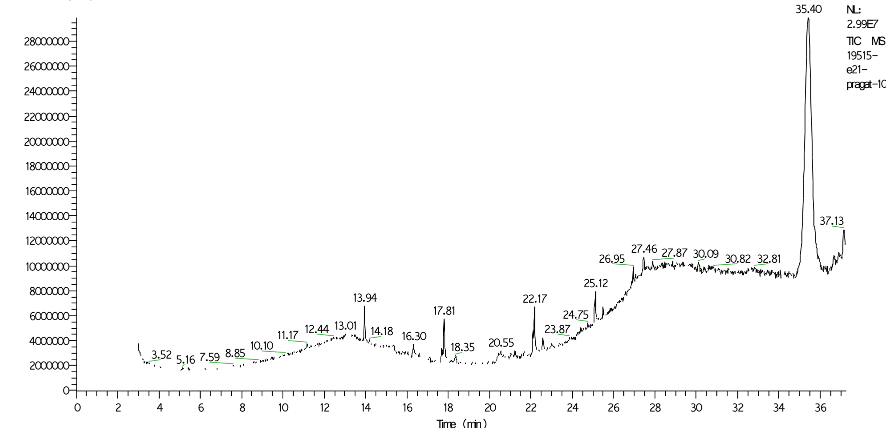


Octadecane, 3-ethyl-5-(2-ethylbutyl)-
Formula $C_{26}H_{54}$, MW 366, CAS# 55282-12-7, Entry# 2126
3-Ethyl-5-(2'-ethylbutyl)octadecane



3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone
Formula $C_{28}H_{25}NO_7$, MW 487, CAS# 127611-84-1, Entry# 35416
3',8,8'-Trimethoxy-3-piperidin-1-yl-2,2'-binaphthyl-1,1',4,4'-tetrone





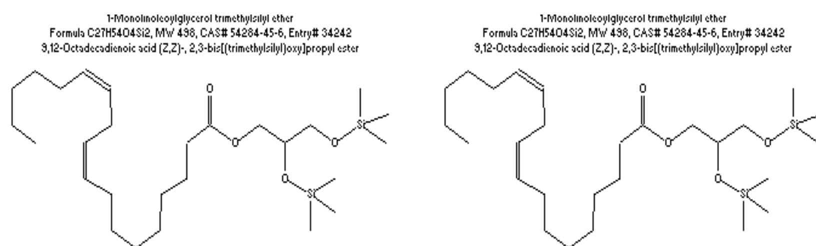


Fig.4 GCMS analysis of Ethyl acetate extract of CS of *Leuconostoc mesenteroides* PL2(3)

PL2 (3), Leuconostoc mesenteroides strain, was shown to produce six different antifungal compounds. As detailed before ethyl acetate extract was subjected to silicon gel column chromatography. About 68 fractions were collected (Fig 3). Antifungal activity was checked for each fraction by performing disc diffusion assay against the test fungus Fusarium oxysporum. The first nine fractions didn't show activity followed by six active fractions followed by 26 inactive fractions after which five fractions showed activity, then nine inactive fractions after which six fractions showed activity and then no activity for remaining fractions.N-eicosane, heptacosane, Octadecane, are the antifungal alkanes described from plant sources but not from microbes.1-nonadecene has been reported from Lactobacillus (Sudhanshu S.B.et al,2019).There are no reports of this alkane from *Leuconostoc mesenteroides* strain. Hepta decanoic acid methyl ester has not been reported from microbial sources.7, 9-Di-tert-butyl-1-oxaspiro (4.5) deca-6, 9-diene-2,8-dione; an antifungal compound has been reported from plant sources (Sharif ,2014), but there are no reports of its production from microbial sources.

The wide variety of antifungal compounds detected from plant isolate of Lactic acid bacteria can be exploited further for applications on preservation of agriculture produce and keep the hazardous effects of chemically synthesized antifungal compounds to a minimum.

CONCLUSION

As observed from the results obtained, Lactic Acid Bacteria isolated from plant sources produce a wide variety of compounds which are antifungal in nature. These compounds can be a good alternative to chemical fungicides and hence be of great help in preservation of agricultural produce by keeping chemical pollution under control.

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