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ORIGINAL ARTICLE **OPEN ACCESS**

Studies on antifungal compounds produced by a plant isolate of Lactic acid Bacteria, *Leuconostoc mesenteroides*

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ABSTRACT
Lactic Acid Bacteria (LAB) is one of the most diverse groups of bacteria. They have been studied extensively for their cultural, biochemical characters and for a wide variety of compounds which they produce. Studies have reported the antagonistic activity of their products against a wide variety of pathogens. Though the LAB are commonly associated with fermented foods and most of the studies done with isolates from fermented foods LAB are found to be associated with various parts of a variety of different plants. The present study was done on a plant isolate of LAB identified as Leuconaostoc mesenteroides. It was seen to produce a wide range of antifungal compounds and showed a very significant inhibitory effect against the test fungus Fusarium sp ,a potential fungal plant pathogen. These compounds were purified by column chromatography, antifungal activity observed for all the fractions and the fractions exhibiting inhibitory action were analysed using GC-MS analysis. A wide variety of antifungal compounds were detected. This property confers biopreservation potential to lactic acid bacteria. The biocontrol potential of lactic acid bacteria can be established for the prevention of fungal infections of agricultural produce specially fruits and vegetables. Thus, living cells or product formulations of antifungal lactic acid bacteria may be prepared and used as an alternative biocontrol technology. The use of these antifungal compounds produced by LAB will reduce the use of chemical fungicides and hence aid in control of chemical pollution. The LAB belong to GRAS (Generally Regarded As Safe) category and hence will not be harmful at the consumers end.
Key Words: Lactic acid Bacteria, Antifungal compounds, Antifungal activity.

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INTRODUCTION

The group that includes the lactic acid bacteria is one of the most diverse groups of bacteria, and these organisms have been characterized extensively by using different techniques. The concept of the group name' Lactic Acid Bacteria' was created for bacteria causing fermentation and coagulation of milk and defines as those which produce lactic acid from lactose. Their growth lowers both, the carbohydrate content of the food that they ferment, and the pH, due to lactic acid production. It is this acidification process which is one of the most desirable side-effects of their growth. The bacteria included in the group are Gram-positive, non-spore forming, cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactic acid bacteria are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins and are considered as 'Generally Recognized As Safe' (GRAS) organisms. Lactic acid bacteria (LAB) convert raw substrates, mainly carbohydrates and proteins, to organic acids, low molecular bioactive peptides, and a significant number of aroma and flavor compounds via their proteolytic activity [1]

Recent studies have reported that health benefits associated with the consumption of these fermented foods is attributable to the presence of LAB compounds with antagonistic activity towards a significant number of pathogenic microorganisms such as organic acids, hydrogen peroxide, bacteriocins, and bioactive peptides [2].

LAB have been isolated from different sources including vegetables and fruits [3], sourdough [4], raw milk [5], dairy products [6], meat products [7] , and fermented beans [8]. Specific LAB strains have demonstrated antimicrobial activity arising from the production of antimicrobial compounds that can be used as natural preservatives in a broad range of food products. With respect to the types of antimicrobial compounds produced by LAB, low molecular peptides are the most important for food preservation. In recent studies, LAB were extensively evaluated for their potential applications as bio-preservation agents to extend the

shelf life of bakery and dairy products, fruits, and vegetables [9-12]. Nevertheless, few studies to date have examined the antifungal properties of LAB-derived low molecular peptides and their survival throughout food processing steps.

Fungal proliferation is a common cause of spoilage in food and feed commodities. Many toxigenic fungi are ubiquitous and secrete low-molecular-weight secondary metabolites known as mycotoxins that can severely affect the health of humans and animals [13]. Mycotoxins are associated with serious human illnesses such as anaemia, immunosuppression and cancers, which threaten public health and may lead to death [14]. Aflatoxins are the most common hazardous mycotoxins that cause both acute and chronic toxicity to humans and livestock. Prevention of fungal growth and production of mycotoxins in foods and animal feeds, are the main strategies to control pathogenic fungi associated illnesses [15]. Preventive strategies have been shown to be more effective for the reduction and elimination of mycotoxins in cereals, nuts and fruits [16] . Chemical preservatives and fungicides are among the primary preventive methods to control fungal growth and limit mycotoxin production, which can extend the shelf life and ensure the safety of food products. For example, the use of fungicides such as carbendazim prevented fungal growth and mycotoxin production in grapes [17]. However, their extensive use has been linked to several health risks, environmental problems and development of resistant strains [18]. Biological control by antagonists such as bacteria, yeasts and fungi can biologically out-compete toxigenic fungi or prevent/reduce their mycotoxin levels1 [19] . Certain safe microorganisms, such as lactic acid bacteria (LAB), can decrease the toxigenic fungi risk and reduce the toxin level to meet safety standards [20, 21]. Other biological approaches depend on the utilization of the naturally produced metabolites (e.g., antimicrobial agents) by safe antagonists that kill the toxigenic fungi or inhibit their growth and production of mycotoxins in food and feed [22].

LAB are promising alternatives to chemical preservatives, as the antimicrobial compounds produced or excreted by LAB have the potential to overcome food-borne pathogens [23]. LAB are natural preservatives that are effective, safe, biodegradable and have additional health benefits. Additionally, LAB have been extensively used as biopreservatives for extending the shelf life of foods and feeds during storage [24-26]. The antifungal compounds produced by LAB include organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl and bacteriocins and bacteriocin-like inhibitory substances [27-29] Although many studies examined the antibacterial compounds produced by LAB, there is only a few studies that describe their antifungal compounds [25, 26, 27]. For example, *Lactococcus lactic* spp. *lactic* ATCC 19435 has been shown to reduce fungal growth and production of ochratoxin A (OCA) in fungal growth media [25, 26]. Antifungal compounds from LAB have been reported as appropriate to control yeasts and moulds that cause food spoilage [12]. Therefore, it is important to seek natural, food-grade antifungal compounds from LAB to control toxigenic fungi and improve the safety, quality and shelf-life stability of food and agricultural products.

The ability to produce several antibacterial and antifungal substances confers biopreservation potential to lactic acid bacteria. Their isolation is reported from vegetables, aerial plant surfaces, pickled cabbage, grass silage, malted cereals and also from soil. They produce antifungal substances, such as cyclic dipeptides, proteinaceous compounds, organic acids, fatty acids and reuterin. The biocontrol potential of lactic acid bacteria is demonstrated in the prevention of fungal infections of fruits, such as apples and grapes. Thus, living cells or product formulations of antifungal lactic acid bacteria may be prepared and used as an alternative biocontrol technology [30].

MATERIAL AND METHODS

Production of crude antifungal preparation

The growth of LAB was suspended in saline to a final turbidity of 1OD (equivalent to 10^9cells /ml). It was inoculated in 500 ml of sterile MRS broth to a concentration of 10^6 cells /ml. The flask was incubated statically for 48 hours at 28^0C.The culture supernatant was prepared by centrifugation at 10,000 rpm for 10 mins followed by filtration through 20 µm syringe filter. (Axiva)

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) were made 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 [29] by using *Fusarium oxysporum*(10^8 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.

2.3 Extraction of antifungal compounds:-

2.3.1 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^{o}C)

↓

Redissolve in double Distilled Water

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

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^{o}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 30^{0}C for 48 hours and observed for zone of inhibition.

2.3.4 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 120^{0}C and then increased to 280^{0}C at 40^{0}C/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.

RESULTS AND DISCUSSION

Potency of antifungal preparation

To determine the potency of the CFS obtained from various isolates. The slope ratio assay was performed where Amphotericin B was used as the standard for comparison. Nystatin and Benzamidazole were also used as standards which are antifungal antibiotics but the test fungus *Fusarium oxysporum* was found resistant to both of them. Similar dilutions of the test compound and the CFS of each isolate were made and assayed by disc diffusion assay and after incubation at 30^{0}C zone diameters were observed .The values obtained were subjected to the slope ratio assay programme and the potencies determined. This can be used for determination of potency of any compound when compared to the standard.

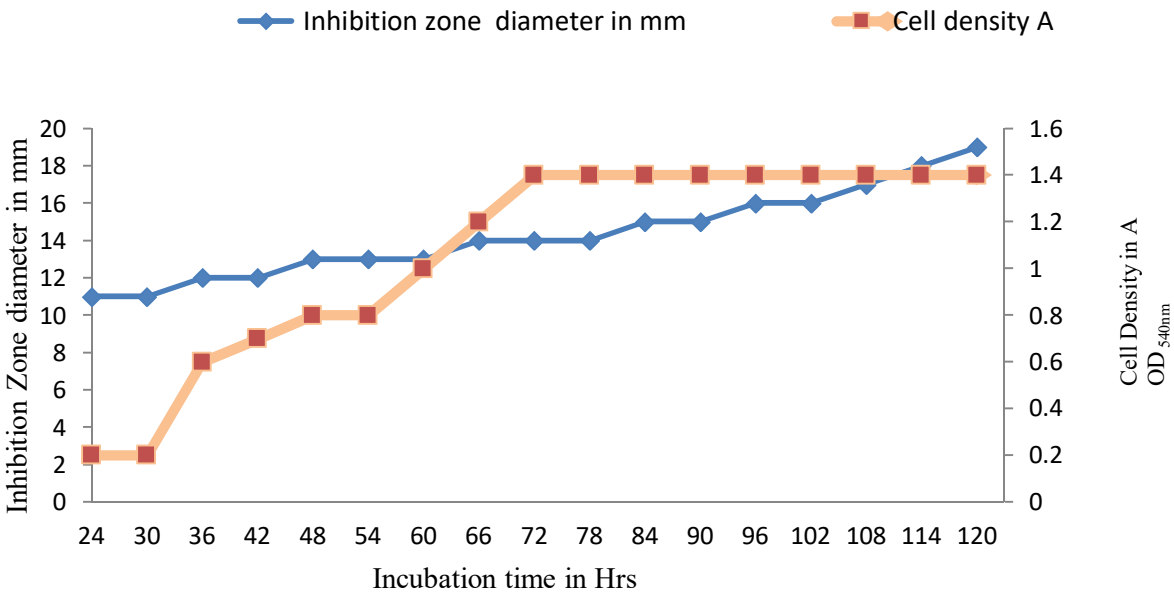


Fig 1: Time course of antifungal production by *Leuconostoc mesenteroides* F3415

Extraction and identification of antifungal compounds

The ethyl acetate extracts showed the best antifungal activity and hence ethyl acetate was the solvent of choice for extraction of antifungal compounds from culture supernatants of all the isolates. After the compounds were extracted in ethyl acetate they were subjected to silica gel column chromatography. Five different combinations of eluents were used to get best possible separation of compounds. About 70 fractions were collected for each extract. Antifungal activity was checked for each fraction by performing disc diffusion assay against the test fungus *Fusarium oxysporum*.

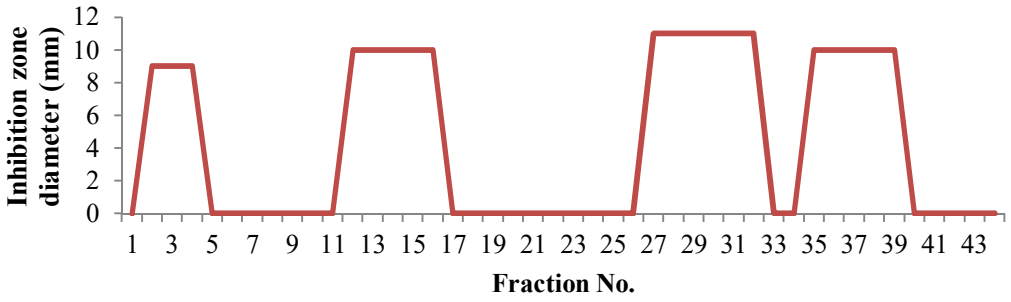
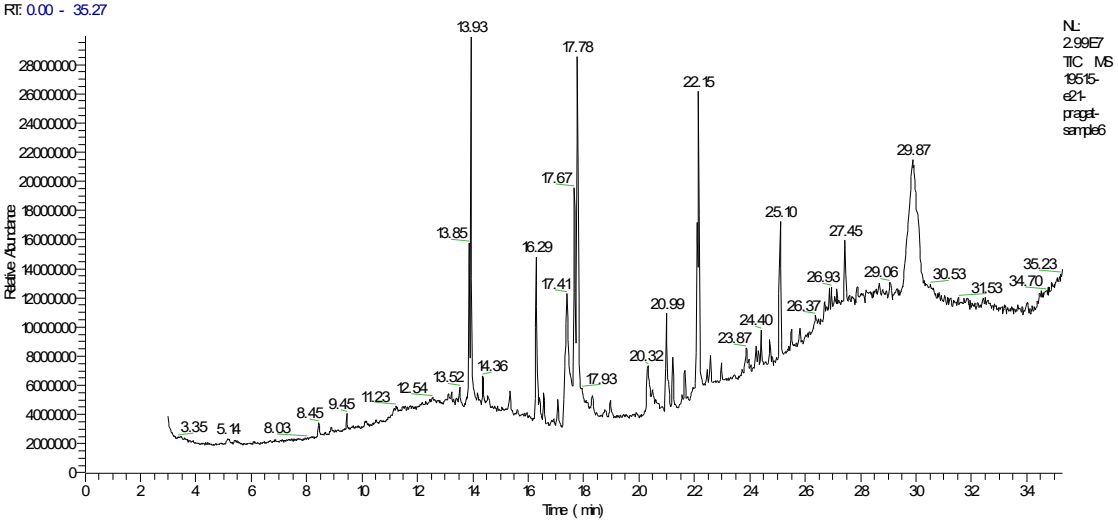
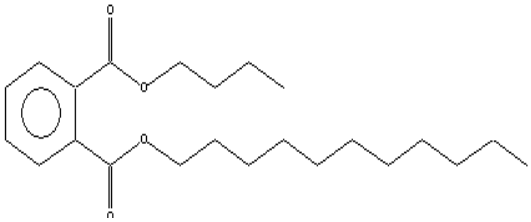


Fig 2: Active fractions collected after silica gel column chromatography of Ethyl acetate extract of CS of *Leuconostoc mesenteroides* (F3415) against Fusarium *oxysporum*.

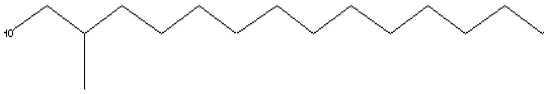


Octadecane
Formula C18H38, MW 254, CAS# 593-45-3, Entry# 5445
n-Octadecane

Phthalic acid, butyl undecyl ester
Formula C23H36O4, MW 376, CAS# NA, Entry# 35332

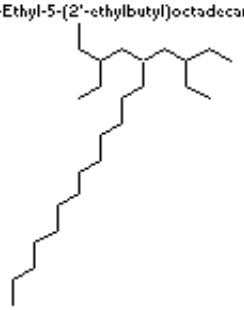


1-Hexadecanol, 2-methyl-
Formula C17H36O, MW 256, CAS# 2490-48-4, Entry# 20717
2-Methylhexadecan-1-ol



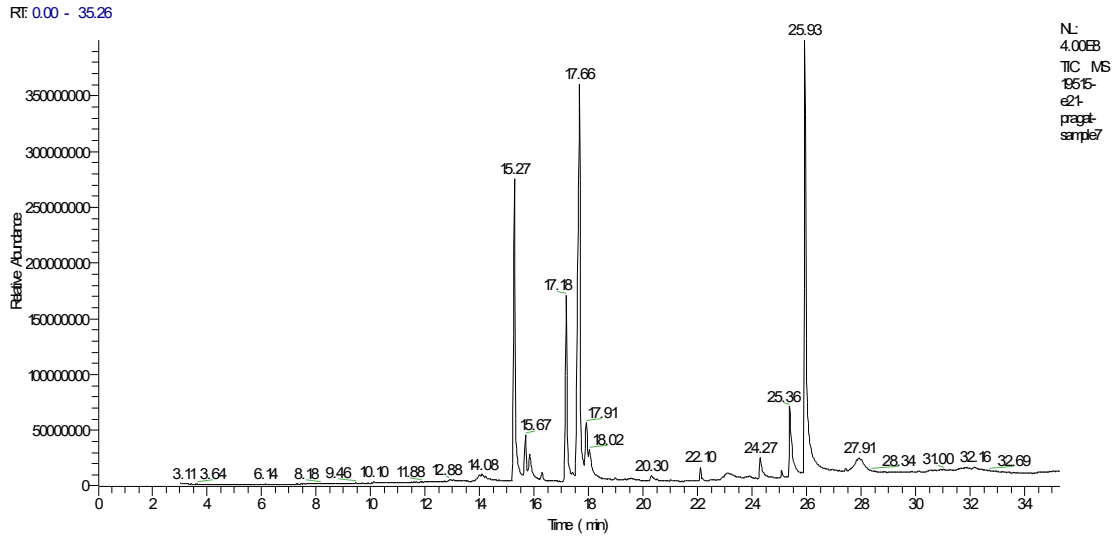
Eicosane
Formula C20H42, MW 282, CAS# 112-95-8, Entry# 5706
n-Eicosane

Octadecane, 3-ethyl-5-(2-ethylbutyl)-
Formula C26H54, MW 366, CAS# 55282-12-7, Entry# 2126
3-Ethyl-5-(2'-ethylbutyl)octadecane



1-Docosanol
Formula C22H46O, MW 326, CAS# 661-19-8, Entry# 2083
Behenic alcohol

Heptacosane
Formula C27H56, MW 380, CAS# 593-49-7, Entry# 5461
n-Heptacosane



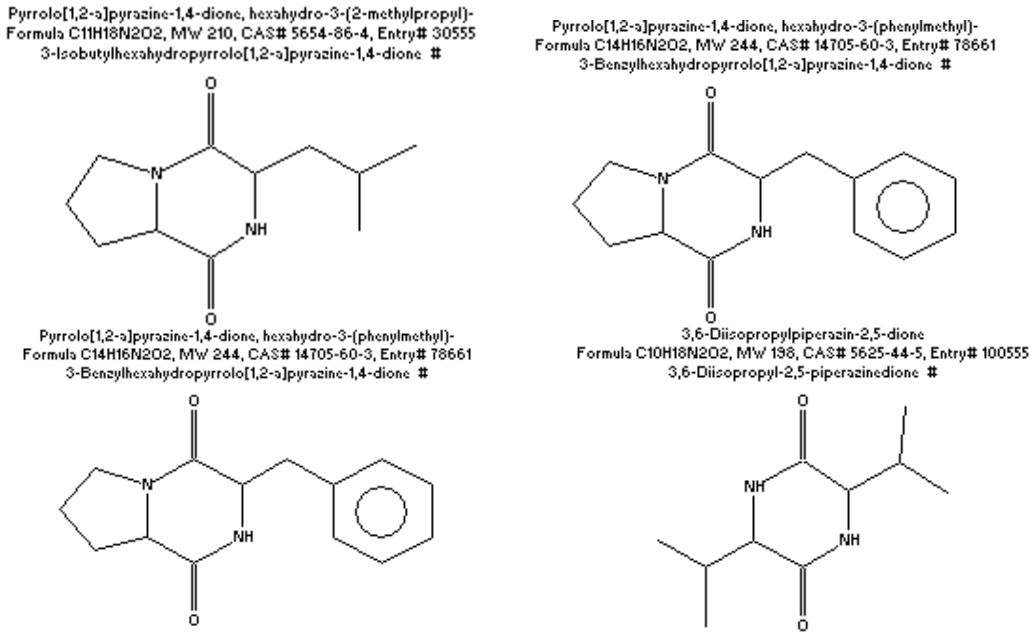


Fig: 2: Extraction and identification of antifungal compounds from *Leuconostoc mesenteroides*

GCMS analysis of Ethyl acetate extract of CS of *Leuconostoc mesenteroides* F3415

For the isolate F3415, *Leuconostoc mesenteroides* the first 12 fractions didn't show any antifungal activity where as the next seven fractions showed good activity. Again the next three fractions showed no activity followed by activity of five fractions. The remaining forty three fractions were without activity. The fractions following in a series which showed antifungal activity were pooled together and subjected to GC analysis and then identified by MS. Seven different compounds were identified for F3415 which are antifungal in nature. Cyclo (pro-Leu) is a cyclic di peptide which has been identified to be produced by *Lactobacillus brevis* [30] , for *Lactobacillus plantarum* (Strom K, et al,2002,), for *L.reuteri* [31] and for *L.casei*, [32]. Production of this cyclic dipeptide has not been reported for *Leuconostoc* genus and hence this becomes the first report of this compound from *Leuconostoc* genus. Similarly another cyclic dipeptide ,Cyclo (phe-pro) was identified which is antifungal in nature and has been reported from *Lactobacillus plantarum* [33, 34] and from *Pediococcus sp.* [35].This cyclic dipeptide again has not been reported for *Leuconostoc* genus and hence this becomes the first report of this compound from *Leuconostoc* genus which was isolated from plant source. Yet another cyclic dipeptide Cyclo(val-val) was identified which is reported to have antifungal activity but there are no reports of this compound having been extracted from any source. This is the first report of the compound which was extracted from any source, from microbial source and more significant for this study from LAB and more importantly LAB from plant source.

N-eicosane and octadecane are the alkanes which were detected for F3415.These two antifungal alkanes have been reported from plant sources but there are no reports from LAB. Similarly docosanol, an alcohol has also been reported from plants as an insect repellant, antiviral and antifungal compound [36] but no reports from LAB. Phthalic acid butyl undecyl esters were also detected. Though this is a known antifungal compound there are no reports of microbial production or production by LAB.

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