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Production Optimization of Antifungal Compound by Lactic Acid Bacterial Isolate from Vineyard

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Abstract
Total 160 bacterial isolates from grape berries and vineyard soil from different sites near Baramati were isolated. On the basis of cultural, Morphological and biochemical characteristics sixteen isolates were considered as lactic acid bacteria (LAB). These sixteen isolates were screened for antifungal activity against *Alternaria alternaria* by dual culture assay. Out of these sixteen isolates, three isolates were found to possess strong antifungal activity while four isolates showed moderate activity. Antifungal activity spectrum of selected LAB against *A. alternata* and *Fusarium graminearum* were determined by agar overlay method. Out of these sixteen isolates, MA100 shows strong antifungal activity against these fungal pathogens. These isolates were further studied for production of antifungal compound, antifungal titer was maximum (1024 AU/ml) when culture was in late log phase (36 h). These isolates were optimized under different Physio-chemical conditions. It was observed that maximum production of antifungal compound (1024 AU/ml) by MA100 in MRS medium of initial pH 7.0 and optimum temperature for production of antifungal compound at 30°C. Production of antifungal activity decreased with increased in salt concentration in medium. Sensitivity of crude antifungal compound was lost after treatment with proteinase-K this indicates, it is protein in nature. Antifungal substance was precipitated maximum at 60% saturation of ammonium sulphate. Total activity & total protein were estimated to determine the specific activity, purification fold & percentage yield. This study revealed that antifungal compound produced by LAB can be used as an efficient biocontrol agent to treat the fungal diseases of grape plant.

Keywords
Antifungal; Lactic acid bacteria; Ammonium sulphate; *Alternaria alternata*

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INTRODUCTION
Grapes are an important agricultural commodity in regions such as Maharashtra in India. The sustainability of vineyards is adversely affected by fungal diseases in grapes. The fungal diseases of grape are *Botrytis cinerea* (Bunch rot), *Erysiphenecator* (powdery mildew), *Botryosphaeria* (canker), *Plasmopara viticola* (downy mildew), *Mycosphaerella*

angulata (Angular leaf spot). Powdery mildew is among the worst grapevine diseases in Maharashtra. The Lactic Acid Bacteria (LAB**)** comprises a diverse group of Gram-positive, non-spore forming bacteria. They occur as cocci or rod and generally catalase negative. They are chemo-organotrophic and grow only in complex media. Hexoses are degraded mainly to lactate (Homofermentative) or to lactate and additional products such as acetate, ethanol, carbon dioxide, formate or succinate (Heterofermentative). [1] LAB are found in fruits (dairy products, fermented meat, sour dough, fermenteted vegetables, silage, beverages), on plants, in sewage, but also in the genital, intestinal and respiratory tract of man and animals. [2] It is well known that rich nutrients like carbohydrates, minerals, nitrogen compound and other substances are necessary for growth of LAB. [3] There are reports on production of antifungal compound by LAB. They are found to produce many antimicrobial products like organic acids, bacteriocins, antibiotics other product like ethanol, hydrogen peroxide, carbon dioxide, diacetyl and acetaldehyde. Recently many reports indicates that LAB also produces antifungal low molecular peptides. [4] LAB are known for the production of antibacterial and antifungal substances. Several LAB produce Reuterin (3-hydroxypropionaldehyde) is an antimicrobial compound produced by *Lactobacillus reuteri* that results from glycerol degradation. [5] There are few reports of antifungal peptides produced by LAB. Magnusson J. [6] described production of a proteinaceous antifungal compound by a *Lact. coryniformis* strain active against several molds and yeasts including *Debaryomyces hanseii* and *Kluyveromyces marxianus*. Several authors have reported the detection of low molecular weight antifungal compounds capable of inhibiting filamentous fungi, but the number of purified and chemically characterized compounds is still low. Coallier-Ascah J. [7] found that *L. lactis* inhibit the growth of *Aspergillus flavus* when grown in co-culture. Thus, LAB was isolated and characterized and the production, optimization, partial purification of antifungal compound by LAB were carried out.

MATERIALS AND METHODS

Sampling:

Collection of aerial parts of the grape plant, grape berries and soil from vineyards were done from different locations around Baramati and Sangali. The sampling sites including Pimpli, Malegaon, Palus & Walwa. During sampling, plant parts i.e. leaves, stem, berries etc. were cut with disinfected scissor and packed in sterile polypropylene bags which were kept in icebox and immediately transported to laboratory.

Processing of Samples:

Samples were cut into small pieces (1x1 cm) with disinfected scissor. The processed sample pieces ten numbers each were suspended into 9 ml sterile MRS broth separately and incubated at 30°C under static microaerophilic conditions for 48 h for enrichment of LAB.

Isolation of LAB:

Isolation of LAB from the sample was performed on MRS agar as well as on LAB selective medium (HiMedia) and the plates were incubated micro-aerobically at 28°C for 48 hours. Colonies were selected on the basis of morphological study.

Screening of LAB for their antifungal activity:

The isolates of LAB were screened for their antifungal activity against the plant fungus pathogen *A. alternata* by overlay technique as described by Strom et al. [8] with slight modification, on MRS agar plates.

A loopful of *A. alternata* culture was inoculated on sterile PDA slant and incubated at 30^{0}C for 6 days for sporulation. Spores from the slant were transferred into a tube containing 10 ml sterile saline with 0.2% Tween 80. The content was vortexed to obtain uniform spore suspension. The LAB isolates were inoculated onto sterile MRS agar plates as 2 cm streaks and plates were incubated under micro aerobic conditions at 30^{0}C for 48 h. These plates were overlaid with semisolid malt extract agar seeded with 10^4 spores/ml of *A. alternata* and incubated aerobically at 30^{0}C for 24 – 72 h.16 isolates of antifungal LAB were tested further for their ability to inhibit wide range of fungi.

Morphology and other microscopic characterization:

The preliminary characterization of isolates included cultural, morphological characteristics, and ability to produce catalase and oxidase. Cultural characteristics of all isolates were studied. Cultural characteristics of typical chalky white colonies appearing on media were noted and growth pattern also observed in liquid

media. The isolates grown on MRS agar plates were examined for cell morphology, Gram character and motility.

Physiological characterization:

Physiological characterization was carried out for the purpose to distinguish between different genera of LAB. [9] This characterization included tolerance to different concentrations of sodium chloride (6.5, 10 and 18%), different temperature (10°C, 30°C and 45°C) and different pH values (4.4, 7 and 9.6). All the tubes were incubated at 28-30°C for 24-48 h. The growth was observed as presence of turbidity. The mode of glucose fermentations was tested in MRS medium with glucose as a carbon source and incubating the fermentation tubes at 30°C for 48 h.

Utilization of carbon sources - The ability of isolates to utilize different carbon sources was tested by incorporating filter sterilized carbon sources (0.2% w/v) into a MRS broth without carbon source and containing bromothymol blue (0.06%w/v) as a pH indicator (alcoholic solution of bromothymol blue), pH 6.8.The carbon compounds included were D-glucose, D-galactose, D-arabinose, ribose, maltose.

Utilization of nitrogen sources -The ability of isolates to utilize different nitrogen sources was tested by incorporating nitrogen sources (0.1% w/v) into a basal medium containing (g/l): Peptone 10, dextrose 20, NaCl 5, K_2HPO_4 0.3 and bromothymol blue 0.06 (alcoholic solution), pH 7.1. The nitrogen sources (0.1% w/v) included alanine, aspartic acid, glutamate, histidine, isolucine, and phenylalanine.

Enzyme activities – Antifungal LAB isolates were tested for production of amylase, catalase, oxidase, chitinase, cellulase and protease.

Antifungal Spectrum of selected LAB isolates:

Sixteen antifungal LAB were tested further for their ability to inhibit the grapes fungal pathogen such as *Fusarium graminearum* and *A. alternata* by overlay technique.

Production optimization of antifungal activity:

Studies on production optimization of antifungal activity - Production optimization of antifungal activity in *MA100* was carried out at varied physico-chemical conditions as well as by incorporating varied concentrations of nutrients in the basal medium. All incubations were under microaerobic environment unless specified. *MA100* was selected for production optimization of antifungal activity from 16 LAB isolates as it showed maximum activity. The sensitive fungal culture, *A. alternata* was used in optimization studies.

Production of antifungal substance in liquid medium by MA100 - *MA100* was studied for its ability to produce active antifungal substance in liquid medium. Overnight grown culture (10^4 cells/ml) was used to inoculate (0.2% v/v) 800 ml MRS broth and incubated under microaerobic conditions, at 30°C for 72 h. Aliquots were withdrawn at 6 h interval and examined for growth (OD at 600 nm), pH and antifungal activity of cell free supernatant (CFS) against most sensitive fungus *A. alternata*. To obtain CFS, 10 ml aliquot was centrifuged at 10000 g for 10 min and the supernatant passed through sterile membrane filter (0.45µm pore size, Millipore). The filtrate was designated as CFS. To determine antifungal activity of CFS by agar well diffusion technique was used.

Antifungal Activity Assay - Antifungal activity was determined in culture extracts by agar well diffusion method. [10] The experiment was carried out in triplicate.

Determination of arbitrary units - An aliquot of CFS obtained at specific time interval during the growth of test LAB in MRS broth was used to study its antifungal activity. The activity was expressed in terms of the arbitrary units (AU) per milliliter of the supernatant, which is reciprocal of the highest dilution at which activity was still obtained. [11]

Effect of types of media - Various media were tested for their ability to support both growth of and the production of antifungal activity. The media tested are MRS broth, Elliker's broth, trypticase soya broth, TYD broth, yeast glucose broth and APT broth. The production of antifungal activity was studied by inoculating *MA100* in each medium

Effect of inoculum size - MRS broth medium was dispensed in 500 ml Erlenmeyer flasks. Overnight grown culture of *MA100* (10^4cells /ml) was inoculated at 0.1, 0.2, 0.3, 0.4, and 0.5 % (v/v) to the fermentation medium and incubated at 30°C for 48 h. CFS obtained was assayed for antifungal activity.

Effect of volume of medium / Space ratio - The effect of volume of medium to space ratio on production of antifungal activity was studied by inoculating test organism into 500ml flasks with varied volume of medium. *MA100* was inoculated (10^4cells/ ml) at 0.2% (v/v) separately into 500 ml flasks with varied volume of medium viz., 100, 150, 200, 250, 300, 350, and 400

ml of sterile MRS broth in 500 ml Erlenmeyer flask and incubated at 30°C for 48 h. CFS of fermented broth was assayed for antifungal activity.

Effect of temperature - The production of antifungal activity (AU/ml) by *MA100* in MRS broth with an initial pH of 6.7 was studied at different temperatures. The incubation temperature was 24°C, 30° C, 37°C and 42°C. Overnight grown culture of *MA100* (10^4cells/ ml) was inoculated at 0.2% (v/v) in MRS broth and incubated for 48h. Antifungal activity of CFS was assayed.

Effect of pH - In order to study effect of pH on production, the experiments were conducted in MRS broth with an initial pH of 5, 6, 7, 8 and 9 obtained by adjusting the pH either with 0.1N HCl or 0.1N NaOH. The media were inoculated with overnight growth of *MA100* (10^4cells/ml) at 0.2% (v/v) and incubated at 30°C for 48h. The antifungal activity of CFS was assayed.

Effect of agitation - MRS broth initially adjusted to pH 6.7 was inoculated with the *MA100* and incubated in rotary incubator shaker (100 rpm) at 30°C for 48h. The control flask was incubated without agitation at the same cultural conditions. The CFS was assayed for antifungal activity

Effect of salt concentration - Effect of NaCl concentration was studied at different concentrations viz., 0, 0.5 .1.0, 1.5 and 2% (v/v) in MRS broth in 100 ml volume separately inoculated with cell suspension of (10^4 CFU/ml) at 0.2% (v/v) and incubated at 30°C for 48h. CFS obtained was assayed for antifungal activity.

Sensitivity of crude Antifungal compound for Proteinase K: The sensitivity of antifungal compound to denaturation by enzyme proteinase K was tested (Munimbazi and Bullerman) [12]. The residual antifungal activity of treated sample was tested by agar well diffusion method against *A. alternata* as target organism.

Purification of antifungal compound:

Ammonium sulphate precipitation- The proteins present in culture supernatant were precipitated by treatment with solid ammonium sulphate to 40%, 60% and 80% saturation. The precipitate obtained was dialyzed against 10 mM phosphate buffer of pH 6.5.

Dialysis of partially purified compound - Partially purified antifungal compound was prepared as described by Bauer et al. [13] with some modification. The protein content and antifungal activity of dialyzed sample and culture supernatant were determined in terms of Arbitrary Unit per ml. Protein concentration of culture supernatant and partially purified compound were determined by Folin-Lowry's method. [14]

RESULTS:

Isolation and characterization of lactic acid bacteria: From each MRS agar plate 10% of the colonies on the basis of morphology were selected for further analysis. In all, 160 colonies were randomly selected with varied morphology on MRS selective agar. Microscopic observations indicated that 47 % of the isolates were rods and 53 % were cocci. Sixteen isolates with antifungal activity were selected for their characterization and identification. Out of 16, seven isolates were rods and remaining cocci. Isolates MA100, MB, MC, M2.2, M2.4, M2.5, M2.6, M2.9, SL2, SB1, SB2, MrsA, SS1, SS2, SF, ST were Gram-positive, non-motile, catalase and oxidase negative, able to grow at 10°C and 45°C and were it also able to grow at pH 9.6 and pH4.4.These 16 Isolate were able to showed growth at 6.5% NaCl but failed to grow at 18 % NaCl. Biochemical characterization was performed as per Bergey's manual of determinative bacteriology 9th edition [15] and Genera of LAB [16] and all biochemical results were compared with those in the manual. All isolates were able to utilize D-Glucose, D-Galactose, D-Arabinose, Ribose, Maltose as a source of carbon and nitrogen utilization data are shown in table 1, These isolates were also studied for utilization of nitrogen sources. Utilization of nitrogen sources by antifungal isolates was also variable. All the isolates could form acid from alanine while isolates MB, MC, M2.4, M2.9, SL2, SS2, ST could not utilize aspartate. All the isolates except M2.2, M2.6, MrsA, SS1, SF were able to utilize histidine as a sole source of nitrogen. The isolates MA100 and M2.5 could not utilize isoleucine while the isolates M2.2, M2.6, MrsA, SS1, SF could not utilize phenylalanine. Enzymatic properties were studied. All isolates were not able to show oxidase, catalase activity, all isolates were unable to show cellulase activity except M2.5 cellulase positive. MA100, MC, M2.4, M2.5, M2.6, M2.9 were Amylase activity. All isolates were shows Protease activity except M2.4 and SL2 shows negative result. All the selected isolates were studied for their ability to ferment glucose under micro aerobic condition. Isolate

MA100, MB, MC, M2.2, M2.4, M2.5, M2.6, SL2, SB1, SB2, MrsA, SS1, SS2, SF were homofermentative Except M2.9, ST were heterofermentative.

Screening of LAB isolate for antifungal activity:

Screening for antifungal activity against *A. alternata* by dual culture assay showed that out of 160 only 16 isolates (10%) were positive for antifungal activity. Inhibitory effects of LAB isolates showed great variation in their antifungal activity against *A. alternata* (Table 2). Three isolates were found to possess strong antifungal activity (inhibition area per bacterial streak was more than 8% of the Petri dish) while four showed moderate (inhibition area per bacterial streak was 3-8 % of Petri dish)

Antifungal activity spectrum:

The antifungal activity spectrum of selected LAB against two grape fungal pathogens was determined by agar overlay method. Out of 16 isolates, M100 showed strong antifungal activity against all fungi, indicating its potential to inhibit the grape fungal pathogen. (Table no.3)

Production optimization of antifungal metabolite:

Time course of production of antifungal activity and growth of MA100

The MRS medium was inoculated with 1x 10^4 cells/ml of *MA100* and incubated at 30^0C. Antifungal activity was detected first at 12 h when culture was in the early logarithmic phase. The antifungal titer was maximum (1024 AU/ml) when the culture was in the late logarithmic phase (36 h) and it was at high until early stationary phase over 12 h. During 72 h of growth, the pH declined from 6.7 to 4.2. The antifungal titer was high when pH of the broth was in the range of 4.8 to 4.5. The decline in antifungal titer was recorded after 48 h, when culture was in stationary phase (72 h) and the AFA titer was 64 AU/ ml when the pH of the broth was 4.2. (fig no.1)

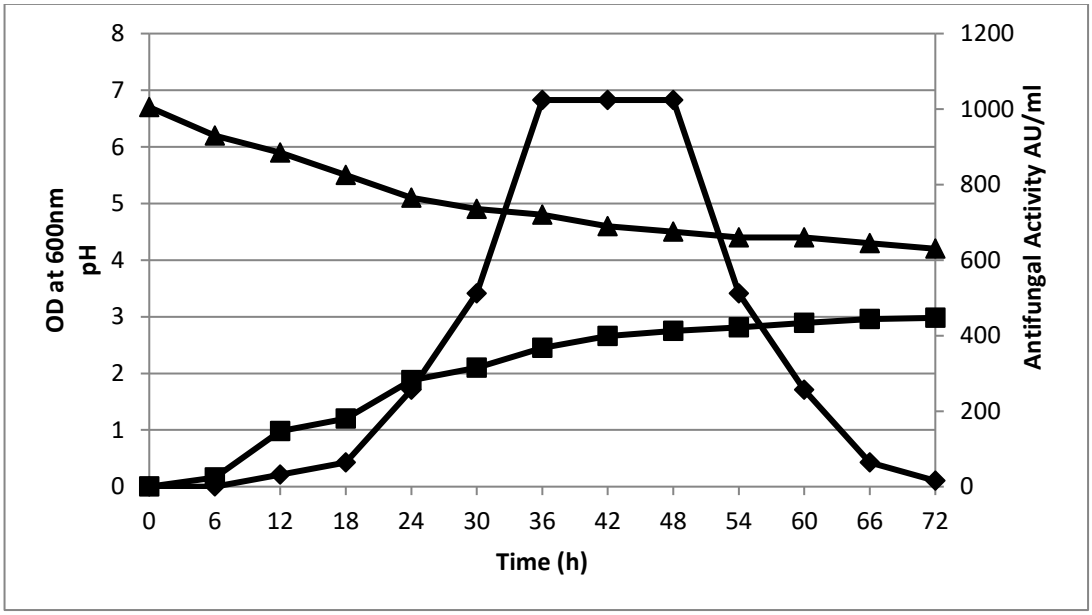

Fig 1. Antifungal compound production by *MA100* in MRS broth for 72 hr. ▲: pH, ■: OD at 600 nm, ◆: Antifungal Activity AU/ml

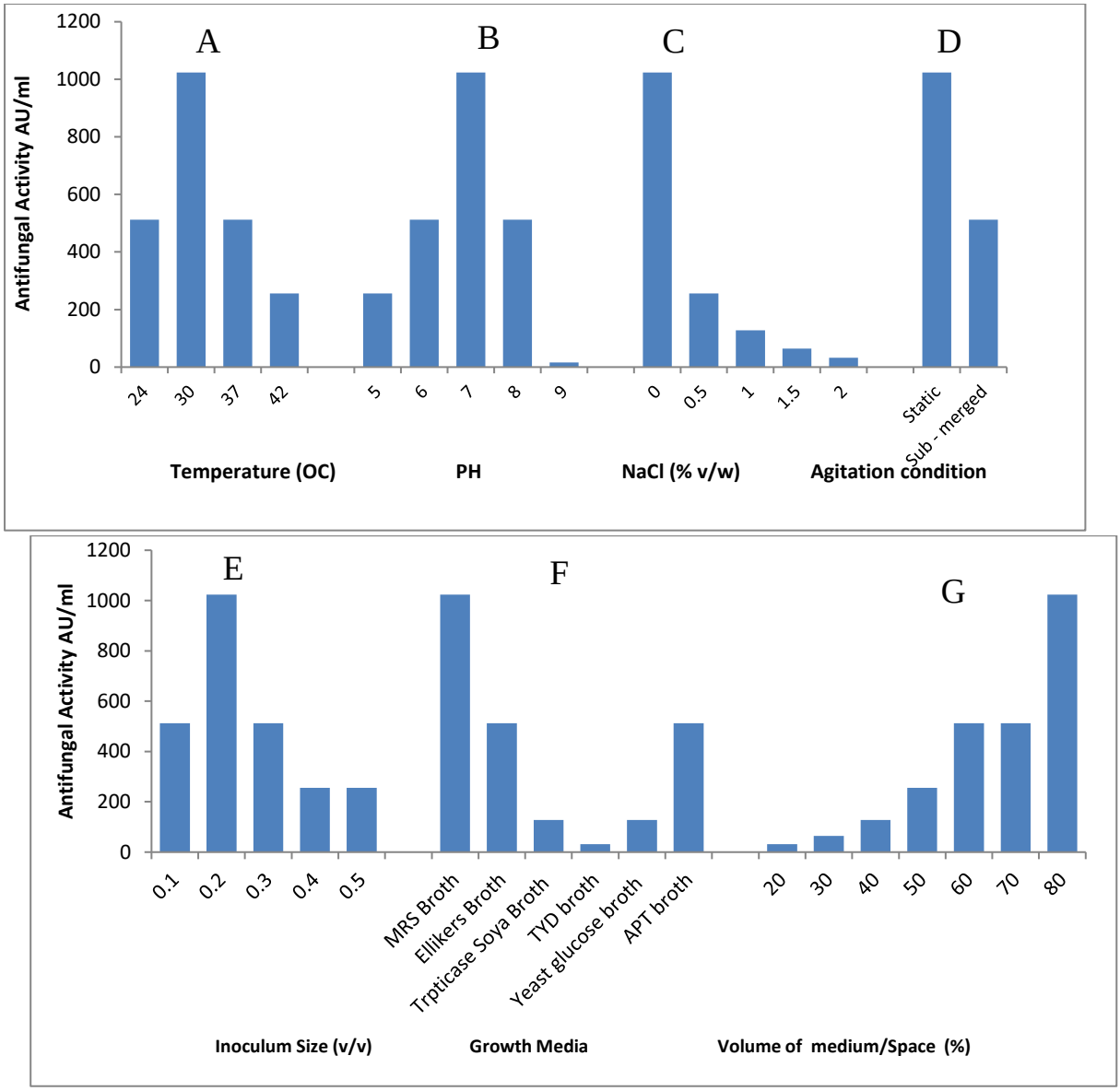

Fig 2: Effect of different parameters on production of antifungal compound. A: Effect of Temperature; B: Effect of pH; C: Effect of NaCl (%v/w); D: Effect of agitation condition; E: Effect of inoculum size (v/v); F: Effect of growth media; G: Effect of volume of medium/space (%)

Table 1: Utilization of various nitrogen sources

Isolate no.	Alanine	Aspartate	Glutamate	Histidine	Isolucine	Phenylamine
MA100	+	+	+	+	-	+
MB	+	-	-	+	+	+
MC	+	-	-	+	+	+
M2.2	+	+	-	-	+	-
M2.4	+	-	-	+	+	+
M2.5	+	+	+	+	-	+
M2.6	+	+	-	-	+	-
M2.9	+	-	-	+	+	+

SL2	+	-	-	+	+	+
SB1	+	+	-	+	+	+
SB2	+	+	-	+	+	+
MrsA	+	+	-	-	+	-
SS1	+	+	-	-	+	-
SS2	+	-	-	+	+	+
SF	+	+	-	-	+	-
ST	+	-	-	+	+	+

Medium used: MRS without nitrogen source + nitrogen source to be tested

Table 2: Screening of LAB for antifungal activity by overlay technique

Isolate	Activity against spoilage fungi *Alternaria alternata*
MA100S, MB, MC	**+++**
M2.2, M2.4, M2.5, M2.6, M2.9	**++**
SL2, SB1, SB2, MrsA, SS1, SS2, ST, SF	**+**

'+' Inhibition area per bacterial streak is of 0.1-3.0% of petridish; '++' Inhibition area per bacterial streak is of 3.0- 8.0% of the petridish; '+++' inhibition area per bacterial streak is of >8.0% of the petridish

Table 3: Activity of LAB isolates against wide range of spoilage fungi.

LAB isolates	*Fusarium graminearum*	*Alternaria alternata*
MA100	++	++
MB	+	++
MC	-	++
M2.2	+++	+++
M2.4	-	+
M2.5	-	+
M2.6	++	+
M2.9	+	+
SL2	+	+
SB1	-	+
SB2	-	+
MrsA	+	++
SS1	+	+
SS2	-	+
SF	-	+
ST	+	+++

+: Average inhibition per bacterial streak; 0.1-3.0% of the petri dish area; ++: Average inhibition per bacterial streak; 3.0- 8.0% of the petri dish area; +++: Average inhibition per bacterial streak; >8.0% of the petri dish area.

Table 4: Purification of Antifungal Compound from culture supernatant of *MA100*.

Ammonium sulphate saturation (%)	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Activity (AUml^{-1})	Total Activity (AU)	Specific Activity (AU mg^{-1})	Fold Purification	Yield (%)
0	100	2.3	230	1024	102400	445.22	1	100
60 (after dialysis)	5	1.50	7.5	1024	5120	682.67	1.53	

Effect of medium type on production of antifungal compound:

MA100 exhibited highest (1024 AU /ml) antifungal activity against *A. alternata* when grown in MRS medium. However, the antifungal activity was 512 AU/ ml in Elliker's broth and APT broth media. Therefore, MRS medium has significant stimulatory effect on production of antifungal compounds as shown in (fig no. 2 F) Hence, MRS medium was used in subsequent experiments.

Effect of inoculum size on production of antifungal compound:

Varied volumes of cell inoculum (10^4 CFU/ml) were used for antifungal compound production. Maximum production (1024 AU/ml) was observed with 0.2% (v/v) inoculum, which decreased with further increase in inoculum size. (fig no. 2 E)

Effect of volume of medium / Space ratio:

The antifungal compound production was carried out in 500 ml Erlenmeyer flasks with ratios varied from 1:5 to 4:5. Maximum antifungal activity production (1024 AU/ml) was observed at 4:5, i.e. 400 ml MRS broth in 500 ml Erlenmeyer flasks. (fig no. 2 G)

Effect of incubation temperature on production of antifungal compound:

The production was carried out at temperatures ranging from 24^0C – 42^0C. The MA100 showed maximum antifungal production (1024 AU/ml) at 30^0C, indicating it to be optimum temperature for production of antifungal activity. For further experiments incubation temperature was maintained at 30^0C (fig no.2 A)

Effect of pH of the medium on production of antifungal compound:

Production media with varied initial pH (5 to 9) were used for antifungal compound production. It was observed that there is maximum production of antifungal compound (1024 AU/ml) in MRS medium of initial pH 7.0. The production was relatively less at pH values above and below 7.0 as shown in (fig no. 2 B)

Effect of agitation on production of antifungal compound:

MA100 produced antifungal activity corresponding to 1024 AU/ml under stationary conditions. Same organism produced 512 AU/ml under submerged conditions. This indicates that *MA100* requires stationary conditions for optimum production of antifungal activity as shown in (fig no. 2 D)

Effect of salt concentration on production of antifungal compound:

The production was carried out using varied concentrations of NaCl (0 - 2%, w/v). There was decrease in antifungal production with increase in salt concentration. The optimum antifungal compound production (1024 AU ml^{-1}) was in the absence of salt. (fig no. 2 C)

Sensitivity of crude Antifungal compound for Proteinase K:

The CFS without enzyme treatment showed inhibition zone diameter of 13mm while inhibition zones were not observed after treatment with enzyme proteinase K. This clearly indicates that there was a loss in the antifungal activity of the CFS after treatment with proteinase K, thus indicating that it is a protein in nature.

Ammonium sulphate precipitation and dialysis:

Ammonium sulphate precipitation method was used to precipitate the proteins present in culture supernatant. The antifungal compound was precipitated maximum at 60% saturation of ammonium sulphate. The total activity and total protein content were estimated to determine the specific activity, fold purification and percentage yield. (Table no.4)

DISCUSSION:

The LAB with antibacterial activity is well documented Kim et al. [17] while less attention has been paid to exploit their antifungal activity [18]. More recently, Magnusson et al. [19]. Isolated antifungal LAB from plant materials. It is obvious from the present investigation that though there is prevalence of large number of LAB on the grape plant, very few of them possess antifungal property. The LAB isolates from grape plant materials seem to be more resistant to stress conditions than those from sea foods [20]. as we observed their growth at extreme pH (4.4 and 9.6) and temperature (10 and 45°C), these are exciting properties in application point of view. Inhibitory activity of LAB against various fungal species is in agreement with previous studies [21, 22, 19]. Nomura et al. [23] isolated *L. lactis* from milk and plants. They found that plant derived *Lactococcus lactis* strains are genetically close to milk derived strains but have various additional capabilities, such as the ability to ferment many additional kinds of carbohydrates and greater tolerance compared with milk derived strains. All the plant derived LAB isolates were unable to grow at 45°C and grew well in the presence of 6.5% NaCl. In the present work the LAB isolates were able to grow at 45°C and similar to earlier finding as regards to their growth in presence of 6.5% salt. Production of antifungal compounds by *Lact. plantarum* is previously reported by few authors [21, 22, 8, 19]. The antifungal compounds produced by *Lact. plantarum* are benzoic acid, methylhydatoin, mevalono-lactone, cyclo (Gly-L-Leu) [21]; Phenyllactic acid, 4-hydroxy phenyllactic acid [20] cyclo (Phe-Pro), cyclo (Phe-OH-Pro), 3-phenyllactic acid [8] Hydroxy fatty acids, phenyllactic acid, cyclo (Phe-Pro) and cyclo (phe-OH-Pro) [19] In the present investigation we found that *MA100* produce maximum antifungal substance in MRS broth as compared to other media used. Drosino et al. [24] found that bacteriocin production by *Leuc. mesenteroides* started during the early growth phase and it was continued until the late active growth phase or the early stationary growth phase, at which the maximum bacteriocin titre was observed. Our results are also similar to the results described by Batish et al. [25] and Drosino et al. [24] In our experiment we also found that production of antifungal activity by *MA100* was growth dependent. Production of antifungal activity started during late log phase and continued until late stationary phase. It was also found that pH optimum for maximal production of AFS (24.16U) by test culture was 6.8 to 7.6. The production of AFS is growth dependent and the environmental / nutritional factors affect growth rate and thus, production of AFS. We also found that AFA was maximally produced if initial pH of medium is adjusted to 7. Roy et al. [26] noted that maximal AFS production by *L. lactis* at 30 °C for 48h. We also found that AFA was maximally produced at 30°C. Aasen et al. [27] was found that bacteriocin activity decreased with increasing in NaCl concentration from 2.5% to 4.0% because of decreased cell biomass. Leroy and De Yuyst [28] reported similar observation. The explanation given by them was that the increase in salt concentration lowers the water activity. Our results indicated that there is reduction in antifungal activity if there is increase in salt concentration. Okkers et al [29] isolated pentosin TV 35 b, a bacteriocin like peptide from *Lact. pentosus* with fungistatic effect on *Candida albicans*. This peptide was purified by ammonium sulphate precipitation. We also found that the antifungal compound lost its activity after its treatment with proteolytic enzymes that indicates its protein nature and was precipitated by ammonium sulphate at 60% saturation.

CONCLUSION:

Antifungal LAB is present on grape plant.10% of total LAB population possessed antifungal property. Thus, this study points out the possibility of using indigenous LAB for production of antifungal compound. Production of antifungal activity was more during late log phase and continued till stationary phase and antifungal titer declined thereafter. Temperature, pH of the medium and inoculum size influences the production of antifungal activity. Production of antifungal activity was decreased with increase in NaCl concentration in the medium. Lower empty head space attributes to higher antifungal production. Antifungal activity was completely lost after treatment with Proteinase K which suggests proteinacious nature of antifungal substance. Antifungal substance was recovered at 60% saturation with ammonium sulphate.

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