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Production and extraction of antifungal compound from *Lactobacillus paracollinoides* FG401

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ABSTRACT
Aim: Production and purification and characterization of the antifungal compounds from *Lactobacillus collinoides* FG401.
Methods and Results: The production for antifungal compound was done in MRS broth. The Antifungal compound was partially purified using ammonium sulphate precipitation followed by column chromatography. The relative molecular weight of purified antifungal compound was checked using SDS-PAGE, found to be between 3KD to 5KD. From IR analysis, the functional groups were determined which may be hydrogen bond, chelated hydrogen bond, alkenes, aromatic alkenes, nitro group, alcohol, phenol, ester, ether or halo-alkenes. The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer. The purified enzyme was quite stable at 40^{o}C and pH range 5.2- 6.8. The antifungal activity of the compound was lost after treatment with proteases.
Conclusions: The antifungal compound produced by the *Lact. collinoides* FG401was found to be produced during late log phase. The purified antifungal compound is a low molecular weight protein and may be a cyclic peptide.
Significance and impact of study: Antifungals from the *Lact. collinoides* can be exploited as biopreservative to prevent post harvest fungal spoilage of agricultural produce.

Keywords: Antifungal, biopreservative, *Lactobacillus collinoide*, spoilage fungi, lactic acid bacteria.

INTRODUCTION

Moulds and Yeasts are commonly involved in the post harvest spoilage of vegetables. Post harvest spoilage can be minimized either by maintaining hygienic conditions or using chemical preservatives. Now a day's consumer demand is increasing for use of ecofriendly biopreservatives instead of chemical preservatives due to their health hazards. Use of LAB in food preservation is known from ancient times. LAB are found on plants and generally recognized as safe, so they can be alternative in biopreservation of vegetables. There are reports on production of antifungal compounds by LAB. They are found to produce many antimicrobial products like organic acids, bacteriocins, antibiotics other products like ethanol, hydrogen peroxide, carbon dioxide, diacetyl and acetaldehyde. Recently many reports indicate that LAB also produces antifungal low molecular peptides (Sathe 2010). In this view, the major objective of this study was to purify antifugal compounds from a potent antifungal *Lact. collinoides* FG401 isolated from fenugreek.

METHODOLOGY

Extraction of antifungal compound

Different solvents were used and tested for the extraction of the antifungal compound from the culture supernatant. The solvents used were n-butanol, n-hexane, ethyl acetate, chloroform and toluene. The solvent was added to the supernatant in 1:1 proportion and mixed in separating funnel by continuous shaking.

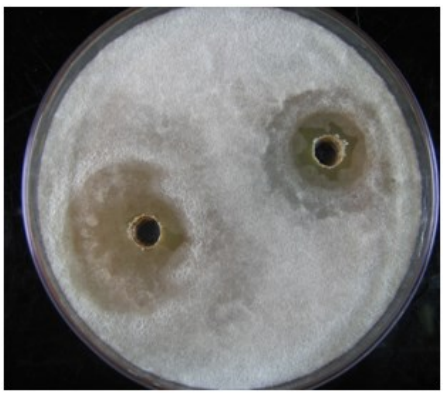

Figure 1 Antifungal activity of CFS (A) and purified antifungal compound (B).

The solvent was evaporated and the residue obtained was dissolved in distilled water and assayed for antifungal activity by agar well diffusion method using *R. stolonifer*.

Ammonium sulphate precipitation

The proteins present in culture supernatant were precipitated by slow addition of solid ammonium sulphate to 40%, 60% and 80% saturation; separately at 4°C with continuous stirring. The mixture was incubated at 4°C for 12 h. Then it was centrifuged at 10,000 rpm for 15 min. at 4°C. The precipitate was recovered in 10 mM phosphate buffer (pH 6.5). This procedure was followed for obtaining precipitate from 40%, 60% and 80% ammonium sulphate saturated solution; separately. Partially purified compound and culture supernatant were tested for antifungal activity and calculated in terms of Arbitrary Unit per ml as described in Sathe *et al.* (2007). Protein concentration of culture supernatant and partially purified compound was determined by Folin-Lowry's method (Lowry *et al*. 1951).

Dialysis of partially purified compound

Partially purified antifungal compound was prepared as described by Bauer *et al.* (2003) with some modification. The ammonium sulphate precipitate was dissolved in 10 mM phosphate buffer of pH 6.5 and dialyzed against the same buffer by using 3 KD molecular weight cutoff size dialysis membrane. Dialysis membrane was activated by treatment with 100 mM $NaHCO_3$ and 10 mM EDTA. Then it was boiled for 10 minutes followed by washing with distilled water twice. The membrane was stored in 4% chloroform. The dialyzed sample was stirred for 24h at 4°C by changing the phosphate buffer 3 times within 24h to remove fatty acids, including residual Tween 80. The protein content and antifungal activity of dialyzed sample were determined.

Column chromatography

Purification of the partially purified compound was carried out by column chromatography using Sephadex G75 having fractionation range 3-100 x 10^{-3}. The column was cleaned using water and rinsed with acetone. After drying the column a small piece of cotton was placed at its bottom. Sephadex G75 gel was then packed in the column by using distilled water as

solvent system. The crude antifungal compound was loaded at the top of the column and eluted using MilliQ water as solvent system. Fractions were collected, where flow rate was adjusted to 1 ml per minute. The ultraviolet absorption spectrum at 280nm and activity of each fraction was detected.

SDS-PAGE electrophoresis

Molecular weight of antifungal compound present in active fractions was determined by SDS-Polyacryl-amide gel electrophoresis using 12% polyacrylamide followed by silver staining (Merril *et al*. 1983).

Infrared and Ultraviolet spectroscopy

The purified sample was kept at 40°C for drying and the powder obtained was subjected to infrared spectroscopic analysis for determination of functional groups. An UV absorption maximum of liquid dialyzed sample was also checked by UV spectrophotometer.

RESULTS

Ammonium sulphate precipitation

The antifungal compound was precipitated maximum at 60% saturation of ammonium sulphate. The precipitate was obtained by centrifuging the ammonium sulphate saturation at 10000rpm for 10 min was dissolved in 10 mM phosphate buffer (pH 6.5). The total activity and total protein content were estimated to determine the specific activity, fold purification and percentage yield (Table 1). After 48h of fermentation the protein content of original culture supernatant was 2.6 mg ml^{-1} and the antifungal activity was 1280 Au ml^{-1}. At 60% saturation of salt, the compound was 5.2 fold purified with 10% yield. The yield decreases due to the loss of the antifungal compound during purification.

Dialysis of the precipitated compound

The salt present in partially purified compound was removed by dialysis using 3 KD cutoff size dialysis membranes (Sigma-Aldrich, USA). Dialysis was done overnight with continuous stirring at 4°C. After dialysis, the activity and percent yield decreases indicate that the other low molecular weight antifungal compounds present (>3KD) may be passed through the dialysis membrane (3 KD cutoff size).

Column chromatography

Further purification of partially purified compound was done by column chromatography using Sephadex G75. The flow rate was adjusted (1ml per minute). Twenty different fractions were collected after loading of crude compound at the top of column, which was eluted with distilled water as solvent system. The absorbance of each fraction was measured at 280 nm. The activity of each fraction was checked by Agar well diffusion assay against *R. stolonifer* as target fungi. Amongst all these 20 fractions, fraction 4, 5, and 6 showed activity.

SDS-PAGE electrophoresis

Fraction obtained from Sephadex G 75 coloumn chromatography as well as dialysed precipitate were run on SDS-PAGE along with the molecular weight markers ranging from 3KD to 97.4KD. The molecular weight of purified antifungal compound is in between 3KD to 5KD.

Table 1 Purification of Antifungal Compound from culture supernatant of *Lactobacillus plantanum* FG401.

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	200	2.6	520	1280	256000	432.3	1	100
40	10	0.60	6	1280	12800	2133.33	4.33	5
60	10	1	10	2560	25600	2560	5.20	10
60 after dialysis	10	0.68	6.8	1280	12800	1882.3	3.82	5

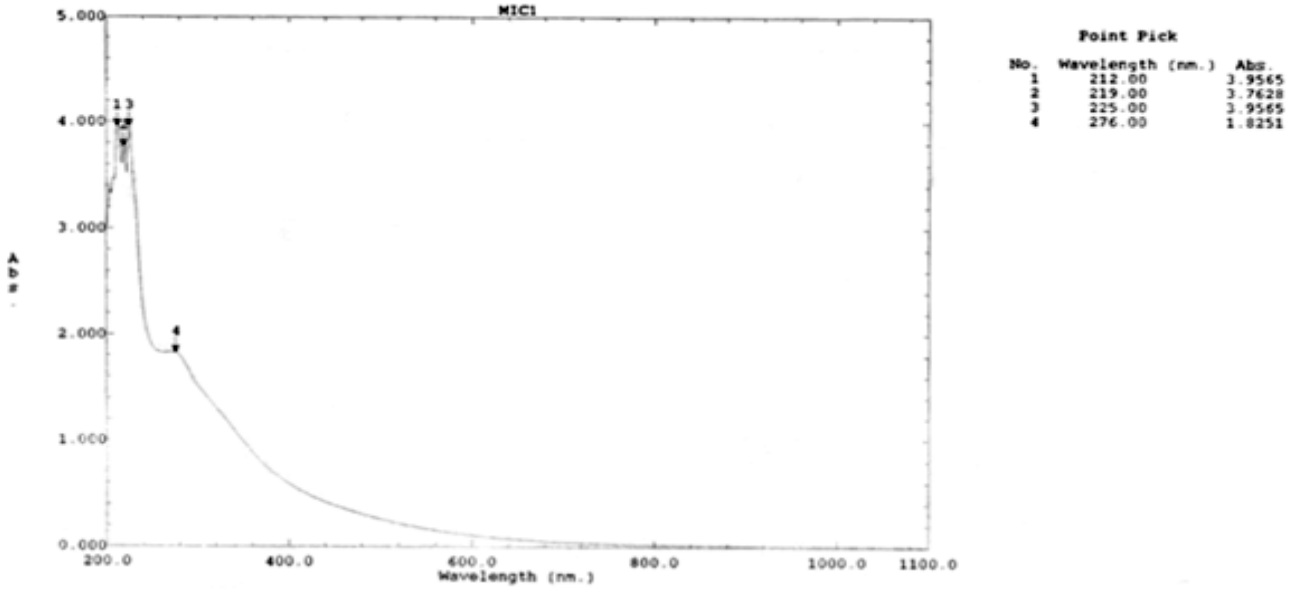

Figure 2 UV Spectrum of Purified antifungal fraction from Sephadex G75 column.

Infrared and UV spectroscopy

The purified sample was kept at 40°C for drying and IR spectrum of the powder obtained was checked for determination of functional groups of the compound**.** From this IR analysis, the functional groups were determined these may be hydrogen bond, chelated hydrogen bond, alkenes, aromatic alkenes, nitro group, alcohol, phenol, ester, ether or halo-alkenes from the frequencies obtained 3288.4, 2970.2, 1643.2, 1527.5, 1417.6, 1091.6, 615.2, respectively in IR spectrum. The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer. Thus, the compound may be a cyclic dipeptide, which absorbs maximally at 215 nm or it may contain Benzene ring, which absorbs maximally at 229 nm (Fig. 2).

DISCUSSION

In the present investigation we found that *Lact. collinoides* produce maximum antifungal substance in MRS broth as compared to other media used.

Okkers et al. (1999) isolated pentosin TV 35 b,a bacteriocin like peptide peptide from *Lact. pentosus* with fungistatic effect on *Candida albicans*. This peptide was purified by ammonium sulphate precipitation, followed by SP- Sepharose cation exchange chromatography. The molecular weight of the peptide was 2.35- 3.4 kDa. Activity of this peptide was lost after treatment with proteiolytic enzymes and the compound was quite stable over a wide range of pH and temperature. Yang and Clausen (2005) found that *L. casei* and *Lact. acidophilus* together inhibit mould growth in liquid culture. The compounds were not identified but found to be heat resistant and showed antifungal activity after neutralization. Lavermicocca et al. (2000) found production of antifungal activity by *Lact. plantarum* and purified antifungals were identified as phenyllactic acid, p-hydroxyphenyllactic acid and palmitic acid. Magnusson *et al*.(2001); Rouse et al. (2008); Batish et al. (1989); Roy *et al*. (1996) reported the production of antifungals by LAB which was due to proteinaceous compound.

We have isolated *Lact. collinoides* from cucumber and it produced protein l compound which is strongly antifungal and showed broad spectrum of activity against vegetable spoilage fungi. Antifungal substance was isolated from the cell free fermented broth by ammonium sulphate precipitation. Attempt to extract antifungal metabolites was failed antifungal activity is precipitated with ammonium sulphate at 60 % saturation. Partially purified compound showed strong antifungal activity. It is observed that antifungal metabolite is of low molecular weight (3

KD). Infra Red analysis indicate that functional groups in the compound may be hydrogen bond, chelated hydrogen bond, alkenes, nitro group, alcohol, phenol, ester, ether or halo alkenes. The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer thus the compound may contain acyclic heteroannular dienes or benzene ring.

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Conflicts of interest: The authors stated that no conflicts of interest.

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