Journal of Microbiology,
Biotechnology and
Food Sciences

Kumar et al. 2012 : 1 (6) 1424-1438

REGULAR ARTICLE

IMPROVEMENT OF ANTIMICROBIAL ACTIVITY OF COMPOUNDS PRODUCED BY *BACILLUS* SP. ASSOCIATED WITH A *RHABDITID* SP. (ENTOMOPATHOGENIC NEMATODE) BY CHANGING CARBON AND NITROGEN SOURCES IN FERMENTATION MEDIA

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ABSTRACT

A specific symbiotic *Bacillus* sp. isolated from a rhabditid entomopathogenic nematode, *Rhabditis (Oscheius)* sp. was found to produce a large number of bioactive compounds. The present study was conducted to determine the effect of carbon and nitrogen sources for the production of antimicrobial substances by *Bacillus* sp. The yield of the crude antimicrobial substances and antimicrobial activity against the test microorganism also differed significantly when carbon and nitrogen sources in the fermentation media were changed. The antimicrobial activity was significantly high in yeast extract plus fructose [*Pencillium expansum* (46.5±2.12 mm) and *Escherichia coli* (42±1.41 mm)] and yeast extract plus maltose followed by meat peptone plus fructose. Antimicrobial activity was significantly reduced with malt extract irrespective of its carbon sources. HPLC analysis of the crude antimicrobial substances revealed different peaks with different retention time indicating that they produced different compounds. When the carbon source was not included in the fermentation media, the antimicrobial production was substantially reduced. The results indicate that carbon source in the fermentation media plays a vital role in the production of antimicrobial substances. It is concluded that yeast extract and fructose as nitrogen and carbon sources produced maximum activity.

Keywords: *Bacillus* sp., carbon and nitrogen, antimicrobial metabolites, fermentation

INTRODUCTION

Despite the critical need for new antibiotics to treat drug resistant infections and other infectious diseases of humans and animals, very few new antibiotics are being developed. At this point, a new antibiotic is required, which is active against resistant bacteria and fungi. In response to microbial resistance, the pharmaceutical industry has initiated to produce a remarkable range of antibiotics **(Luzhetskyy *et al.,* 2007)**. Keeping in view these facts, there is an urgent need to discover new antibiotics to treat multidrug-resistant infections. Microorganisms are a virtually unlimited source of novel chemical structures with many potential therapeutic applications **(Behal, 2000).** Microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. Generally, the reason why they produce such metabolites is not known, but it is believed that many of these metabolites may act as chemical defense of microbes competing for substrates **(Gallo *et al.,* 2004).** Although several hundreds of compounds with antibiotic activity have been isolated from microorganisms over the years, but only a few of them are clinically useful **(Thomashow *et al.,* 2008).** The outstanding role of microorganisms in the production of antibiotics is notorious. At present, with 1% of the microbial world having been explored, the advances in techniques for microbial cultivation and extraction of nucleic acids from soil and marine habitats are allowing access to a vast untapped reservoir of genetic and metabolic diversity **(Sanchez and Olson, 2005).**

It is well known that 30-40% of the production cost of antibiotics is taken up by the cost of growth medium **(Barrios-Gonzalez *et al.,* 2005).** Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture **(Papagianni, 2004).** Antibiotic formation usually occurs during the late growth phase of the producing microorganism. The temporal nature of their formation is certainly genetic, but the expression can be influenced greatly by environmental manipulations. Therefore, synthesis of antibiotics is often brought on by exhaustion of a nutrient, addition of an inducer and/or by a decrease in growth rate **(Bibb, 2005).** However, the production of antimicrobial substances depends upon the substrate medium for their optimal growth, temperature, pH and the concentration of nutrients in the medium **(Leifert *et al.,* 1995).** Carbon as a part of an ingredient in the medium is

required for bacterial growth and to enhance the production of antimicrobial substances. Antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources **(El-Banna, 2006)**. A balanced ingredient in the medium as nutrition for bacterial growth and production of antimicrobial substances is important. Their synthesis can be influenced by manipulating the type and concentration of nutrients formulating the culture media. Among them, the effect of the carbon source has been the subject of continuous studies by both industry and research groups **(Sanchez *et al.*, 2010)**.

Entomopathogenic nematodes (EPN) are biological control agents of insect pests and are symbiotically associated with specific bacteria pathogenic to insects. EPN infested cadaver does not purify. This is being achieved by the production of a number of bioactive compounds by the bacteria which can suppress invading microorganisms of the cadaver from within and outside the environment. The study of the bioactive compounds produced by the EPN had already been initiated in different laboratories around the world **(Akhurst and Dunphy, 1982; Chen *et al.*, 1994; Maxwell *et al.*, 1994; Li *et al.*, 1995a; Li *et al.*, 1995b)**.

The objective of our study was to find out the effect of carbon and nitrogen sources in the fermentation media on antimicrobial substance production and to find out the ideal carbon and nitrogen sources for bacteria to produce the antimicrobial substances against bacteria and fungi.

MATERIAL AND METHODS

All chemicals used for extraction and purification were of AR grade (Merck, Mumbai, India). Nutrient agar, Mueller Hinton Agar (MHA), nutrient broth, potato dextrose agar, potato dextrose broth and six nitrogen sources (meat peptone, beef extract, yeast extract, tryptone, meat infusion extract and malt extract) were purchased from the Himedia Laboratories Limited, Mumbai, India. The carbon sources used are fructose, maltose, dextrose, mannitol, sucrose and lactose were purchased from the SRL Laboratories Limited, Mumbai.

Test microorganisms

Test pathogens were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, which included Gram positive bacteria, *Bacillus subtilis* MTCC 2756*;* Gram negative bacteria *Escherichia coli* MTCC

2622; agriculturally important fungi *Pencillium expansum* (local isolate) and medically important yeast *Candida albicans* MTCC 277. Nutrient agar was used for subculturing the bacteria and potato dextrose agar slants for subculturing the fungi and yeast.

The antimicrobial substance producing bacteria was isolated from an entomopathogenic, *Rhabditis (Oscheius)* sp. resembling *Rhabditis* isolate Tumian 2007 at D2 and D3 (nucleotide sequence region) expansion segments of 28S rDNA. Nucleotide sequence of the 16S rDNA of the bacteria associated with the nematode exhibited high similarity to the *Bacillus* 16S rDNA genes. The 16S rDNA sequence was deposited with NCBI (accession number AHQ200404). The bacteria has been deposited with IMTECH, Chandigarh (accession number is MTCC 5234).

Fermentation media preparation

Bacterial isolate was inoculated into the liquid medium. The liquid media was prepared with different carbon sources (fructose, maltose, dextrose, mannitol, sucrose and lactose) as the first factor nutrient and different sources of nitrogen (meat peptone, beef extract, yeast extract, tryptone, meat infusion extract and malt extract) as the second nutrient sources. The liquid media composed of (g/L): carbon source (10.0), nitrogen source (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4$ (1.0), NaCl (2.0), Na_2SO_4 (1.0). The media pH was adjusted to 7.0 before autoclaving using NaOH or HCl solution.

Hundred mL aliquots of each media containing one each of different carbon and nitrogen sources were dispensed separately in 250 mL Erlenmeyer flasks and this was inoculated with a loop full of the bacterial culture. The flasks were incubated in a controlled environment, gyrorotatory shaker (150 rpm at 30 $^{\circ}$C in darkness for 24 – 48 hours. When the optical density of the culture at 600 nm was approx 1.7 (AU), the bacterial cultures were transferred into 400 mL sterile medium and incubated in a gyrorotatory shaker (150 rpm) at 30 $^{\circ}$C in darkness for 96 hours. The culture media were then centrifuged (10,000 rpm, 20 min, 4 $^{\circ}$C) followed by filtration through a 0.45µM filter, to obtain cell free culture filtrate.

Preparation of crude organic extract

The cell free culture filtrate (500 mL) was neutralized with 1 N HCl and extracted with an equal volume (500 mL) of ethyl acetate thrice. The ethyl acetate extracts were combined,

dried over anhydrous sodium sulphate, and concentrated using a rotary flash evaporator at 30 °C to obtain the crude extract.

Determination of antibacterial activity

Antibacterial activity was determined following the paper-disc diffusion assay **(Lippert *et al.*, 2003)**. The test bacteria were cultured in Nutrient agar (Himedia, Mumbai) and incubated at 37 °C for 18 hours and were suspended in saline solution (0.85 % NaCl) and adjusted to a turbidity of 0.5 McFarland standards (10^6 CFU.ML^{-1}). The suspension was used to inoculate on MHA plates. Sterile paper discs (6.0 mm diameter, Whatman antibiotic assay disc) impregnated with 1 mg.ML^{-1} concentration of different crude extracts was placed on the surface of the medium using alcohol-flame-sterilized forceps. Petri-dishes were kept at room temperature for 1 hours to allow the diffusion of the crude extracts and then inverted and incubated for 18-24 hours at 37 °C. The diameter of inhibition zone was measured in mm. Ciprofloxacin (5 µg.ML^{1}) (Himedia) was used as a positive reference standard to determine the sensitivity of the strains.

Determination of antifungal activity.

Antifungal activity was determined using the paper-disc diffusion assay **(CLSI 2008)**. The fungal cultures were swabbed on the surface of the potato dextrose agar (PDA) medium (Himedia, Mumbai). Paper disc (6 mm) was placed on the surface of the seeded PDA plates and 25 µg.$ML^{-1}$of each crude was added and air dried in laminar air flow. The diameter of zone of clearance on the PDA medium was measured at 2 days after incubation.

HPLC analysis of crude extracts.

The crude ethyl acetate extracts were analyzed by analytical HPLC (Shimadzu, Japan). Sample (20 µL) was injected into a C18 column (250 mm X 4.6 mm X 5 mm). The flow rate was 1 ml.Min^{-1} and the mobile phase was methanol: water (50:50). Constituents eluting from the column was detected at 220 nm using a Shimadzu UV–VIS detector.

Statistical analysis.

All statistical analyses were performed with SPSS (Version 17.0; SPSS, Inc., Chicago, IL, USA). Data for time kill analysis was presented as means ± standard deviations. Statistical significance was defined as $p< 0.05$.

RESULTS AND DISCUSSION

Yield of crude extract

Six different carbon and nitrogen sources at a concentration of 1% were used in the present study to find out the ideal fermentation media having enhanced yield and antimicrobial activity. A total of 42 different combinations were used in the present study. There was a high degree of variation in the yield of crude extract when the different carbon and nitrogen sources were tested in the fermentation medium (Fig 1). The yield was the highest in the beef extract and fructose (921±6.39 $mg.L^{-1}$) followed by yeast extract and maltose (820±8.48 $mg.L^{-1}$). The lowest crude yield extract was recorded for malt extract and maltose (38.5±3.53 $mg.L^{-1}$). The yield of crude extract was considerably higher when the media consisted of carbon and nitrogen sources and low yield was recorded when the nitrogen source alone was used in the fermentation media (Fig 2). The yield of crude extract was higher when dextrose was used as carbon source for all the nitrogen sources followed by maltose and fructose

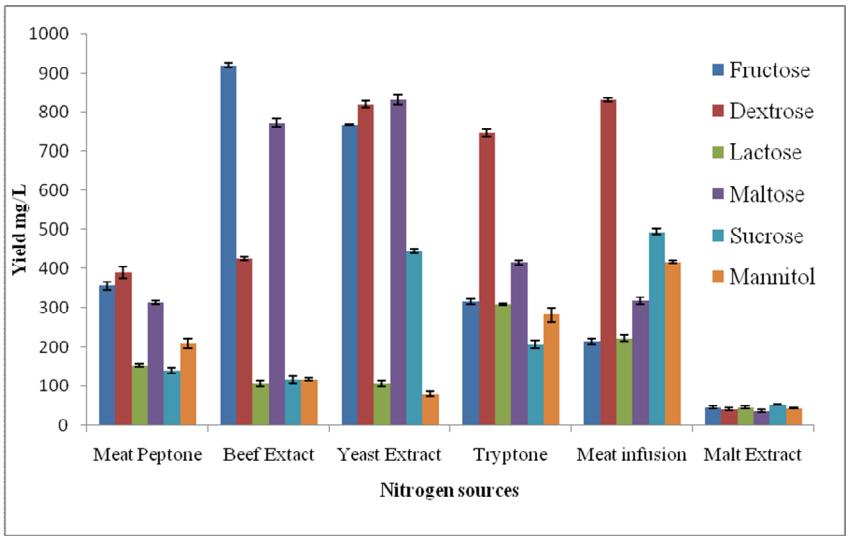

Figure 1 Yield of crude extract under different Carbon and Nitrogen sources

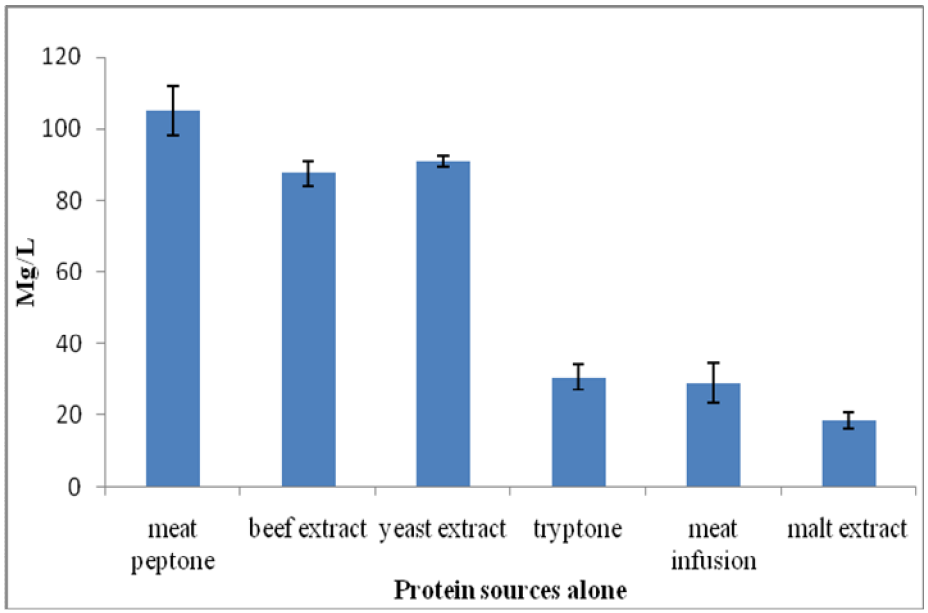

Figure 2 Effect of nitrogen sources alone on the crude extract production

Antimicrobial activity of extract

There was a high degree of variation in the level of antimicrobial activity against the test microbes when the different carbon and nitrogen sources were added in the fermentation medium (Fig.3. A-F). The antimicrobial activity was higher in the combination of yeast extract and fructose and highest activity of this combination was recorded against *P. expansum* (46.5±2.12 mm) and *E. coli* (42±1.41 mm) (Fig. 3C and Fig 4). Yeast extract and maltose also recorded a similar pattern of activity against the test microorganisms. Beef extract and fructose also recorded good antimicrobial activity and highest activity of this combination was recorded against *B. subtilis* (37±2.82 mm) and *E. coli* (31.5±0.7 mm) (Fig. 3 A and B). Meat peptone and dextrose recorded good antimicrobial activity and highest activity was recorded against *B. subtilis* (36±1.41 mm). Antimicrobial activity was considerably decreased when nitrogen sources alone were used in the fermentation media.

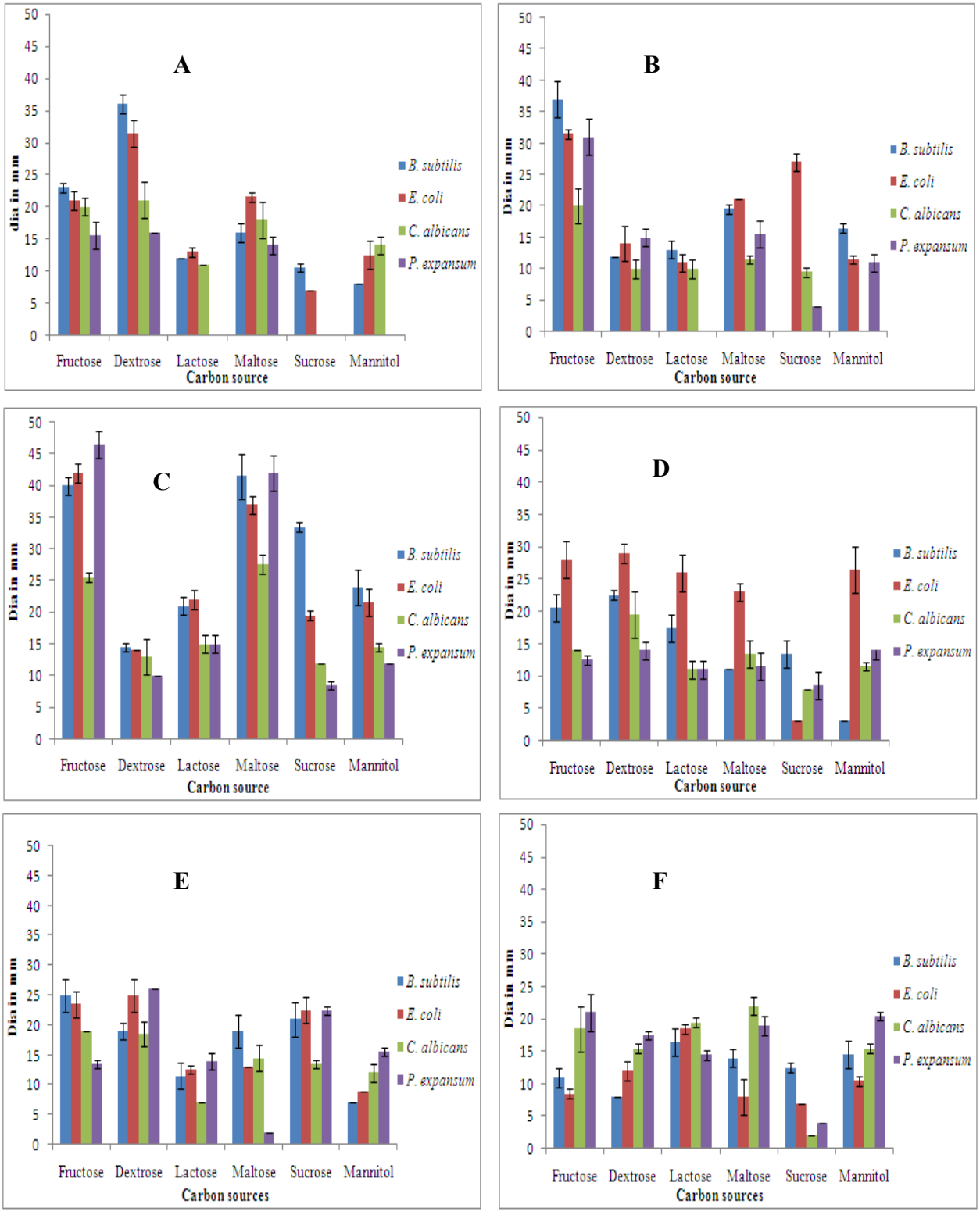

Figure 3 Antimicrobial activity of carbon and nitrogen sources: A-Meat peptone and carbon sources, B- Beef extract and carbon sources, C-Yeast extract and carbon sources, D-Tryptone and carbon sources, E-Meat infusion and carbon sources, F- Malt extract and carbon sources

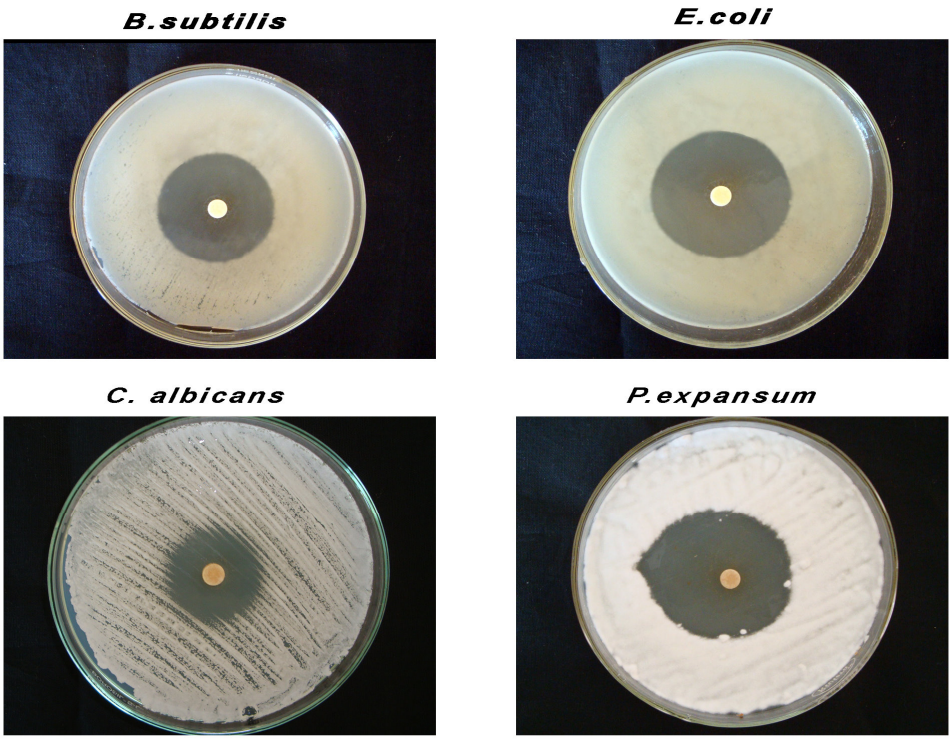

Figure 4 Antimicrobial activity of yeast extract plus fructose by agar disc diffusion method

The result of the present study clearly indicated that an ideal carbon and nitrogen sources are needed for better antimicrobial activity and best carbon source in our study was fructose and nitrogen source was yeast extract. Carbon sources in the media play a very critical role in the production of antimicrobial substances by the bacteria.

HPLC analysis

In HPLC analysis yeast extract and fructose recorded 59 compounds and retention time (Rt) ranges from 0.46 to 33.51 min (Fig. 5A). Even though the combination of yeast extract with fructose and maltose recorded almost similar bioactivity, yeast extract plus maltose recorded only 42 compounds (Fig. 5B). Compounds at 2.64 and 8.04 min were same in both combinations. This reveals that these two peaks play an important role in the bioactivity. But meat peptone plus fructose recorded only 35 compounds (Fig. 5C) and comparing with Rt of this combination gives the clear picture that this produced different compounds. The retention time of the peaks with different combinations was quite different in each combination indicating the production of different molecules.

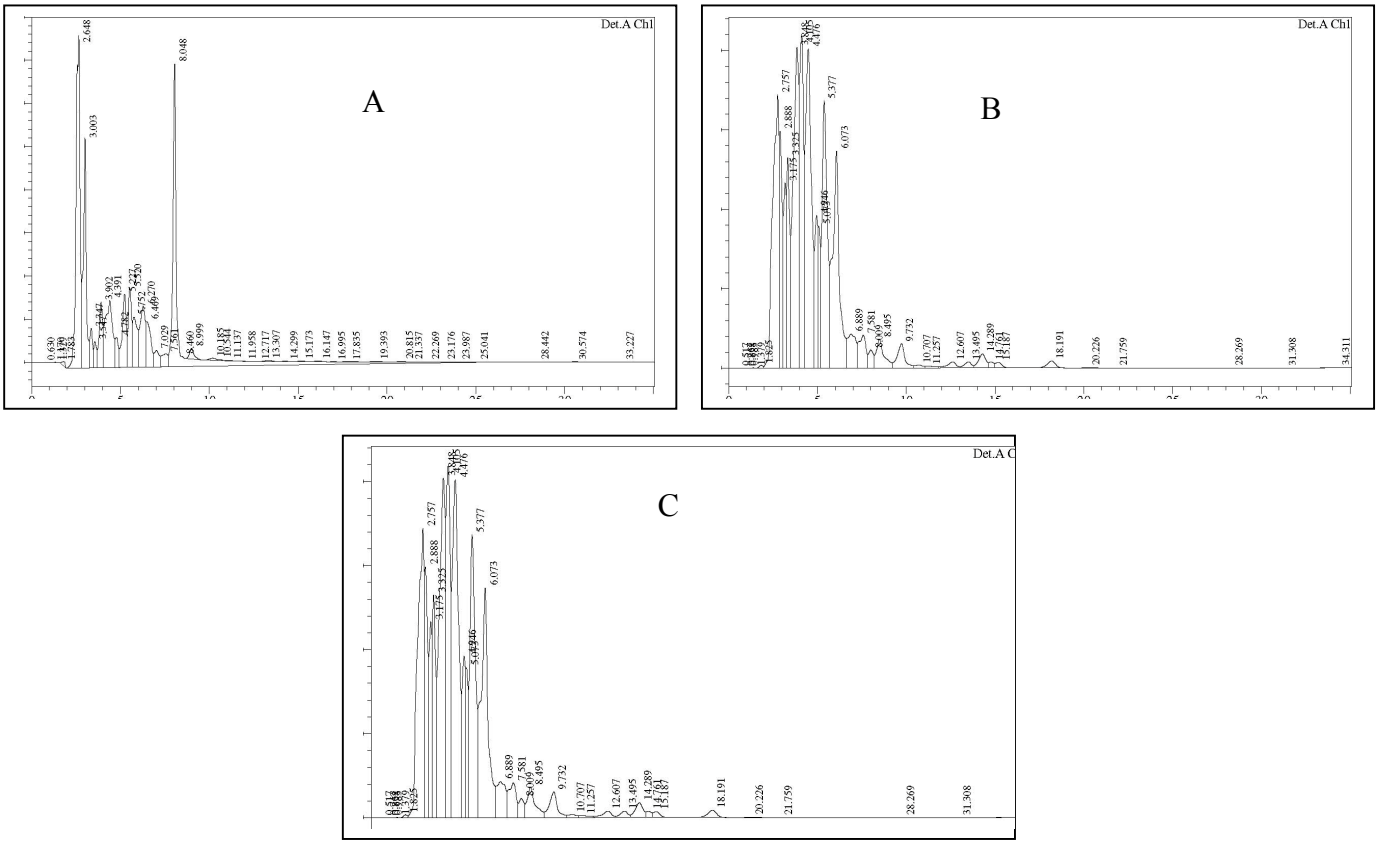

Figure 5 HPLC chromatogram of three combinations on reversed-phase C18 column (LC-20AD). Samples of 20 µL were injected to a column (250 mm ×4.6 mm × 5 mm), eluted with methanol:water (50:50) (A) yeast extract plus fructose (B) yeast extract plus maltose (C) meat peptone plus fructose

DISCUSSION

For the past five decades, the need for new antibiotics has been met largely by semisynthetic tailoring of natural product scaffolds discovered in the middle of the 20th century **(Luzhetskyy *et al.*, 2007)**. More recently, however, advances in technology have sparked resurgence in the discovery of natural product antibiotics from microbial sources. This has resulted in several newly discovered antibiotics with unique scaffolds and/or novel mechanisms of action, with the potential to form a basis for new antibiotic classes addressing bacterial targets that are currently underexploited. Natural products represent the traditional source of new drug candidates **(Luzhetskyy *et al.*, 2007)**. Formation of antibiotics is also regulated by nutrients (such as nitrogen, phosphorous and carbon source), metals, growth rate, feedback control and enzyme inactivation **(Sanchez and Demain, 2002).** Among these nutrients, the effect of carbon and nitrogen source on antibiotic production has been the

subject of continuous study for both industry and research groups, not only from fermentation but also from biochemical and molecular biological stand points. The carbon and nitrogen sources are the important constituents to be considered which are reported to have highly influenced on the antibiotic production by nematode associated bacteria **(Yang *et al.,* 2006; Wang *et al.,* 2008).**

Different carbon sources, like dextrose **(Rizk and Metwally, 2007**), lactose **(Petersen *et al.,* 1994**), sucrose **(Charkrabarti and Chandra, 1982**), fructose **(James and Edwards, 1988**), starch **(Kotake *et al.,* 1992**) and glycerol **(Bhattacharyya *et al.,* 1998**) have been reported to be suitable for production of secondary metabolites in different microorganisms. The choice of carbon sources greatly influenced secondary metabolism and therefore antibiotic production **(Martin and Demain, 1978; Roitman *et al.,* 1990 & El-Benna, 2006)**. Variations in the fermentation environment often result in an alteration in antibiotic production. The alteration involves changes both in yields and in the composition of the substances **(El-Benna, 2006)**. In our study variation in nitrogen and carbon sources resulted in the variation in the antimicrobial activity which indicated that the composition of the secondary metabolites produced by the bacterium could be changed. It has been known that cultivation parameters are critical to the secondary metabolites produced by microorganisms. Even small changes in the culture medium may not only impact the quantity of certain compounds but also the general metabolic profile of microorganisms **(Scherlach and Hertweck, 2009).** Manipulating nutritional or environmental factors can promote the biosynthesis of secondary metabolites and thus facilitate the discovery of new natural products. Highest antimicrobial activity was recorded for fructose and maltose in combination with yeast extract. Thus regulation by the carbon source depends on the rapid utilization of the preferred carbon source.

Sometimes quickly metabolized substrate such as glucose may achieve maximum cell growth rates, but is known to inhibit the production of many bioactive secondary metabolites **(El-Benna, 2006)**. In the present study also dextrose recorded significantly higher yield of crude extract with low antimicrobial activity except with meat peptone. In our study antimicrobial activity was considerably enhanced when fructose was used as carbon source and this result was supported by **(Bhattacharyya *et al.,* 1998**) who reported that the carbon source needed for maximal yield of the antibiotic production seems to be different among bacterial strains.

The nature of the nitrogen source used has a notable effect on the production of the antimicrobial metabolite in the bacterium. In our study yeast extract recorded significant

effect on the antimicrobial production followed by meat peptone and beef extract. Depending on the biosynthetic pathways involved, nitrogen sources may significantly affect antibiotic formation **(Gesheva *et al.*, 2005**). The results of the present study indicated that nutrient in the fermentation media plays an important role in the onset and intensity of secondary metabolites, not only because limiting the supply of an essential nutrient is an effective means of restricting growth but also because the choice of limiting nutrient can have specific metabolic and regulatory effects **(Doull and Vining, 1990).**

CONCLUSION

The results of antimicrobial susceptibility tests indicated that antimicrobial metabolite obtained from the bacterium may be produced optimally in the presence of fructose as carbon source and yeast extract as a nitrogen source. This work will be useful for the development of *Bacillus sp.* cultivation processes for efficient antibiotic production on a large scale, and for the development of more advanced control strategies on plant and animal diseases.

Acknowledgements: We are grateful to Department of Science and Technology-Drugs and Pharmaceutical Research Programme (DST- DPRP), Government of India for funding. We thank the Director, CTCRI, for providing facilities for the work.

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