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Influence of Six Carbon Sources with Yeast Extract on Antimicrobial Metabolite Production by Bacterium Associated with Entomopathogenic Nematode

Sasidharan Nishanth Kumar* • **Bala Nambisan** • **R. Ramya** • **Chellapan Mohandas**

Division of Crop Protection and Division of Crop Utilization, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram 695017, India

Corresponding author: * **nishanthctcri@gmail.com**

ABSTRACT

A specific symbiotic *Bacillus* species isolated from a rhabditid entomopathogenic nematode, *Rhabditis (Oscheius)* sp. was found to produce a number of bioactive compounds. The present study was conducted to determine the effect of six different carbon sources in combination with yeast extract on the production of antimicrobial substances by *Bacillus* sp. The yield of crude antimicrobial substances and antimicrobial activity against the test microorganism also differed significantly when the carbon sources in the fermentation media were changed. The highest yield was recorded for maltose plus yeast extract (836 mg/L). The antimicrobial activity was significantly higher in yeast extract plus fructose [*Pencillium expansum* (46.5 ± 2.12 mm) and *Escherichia coli* (42.00 mm)] than yeast extract plus other carbon souces used in the study. Antimicrobial activity was significantly reduced in yeast extract plus glucose. HPLC analysis of the crude antimicrobial substances revealed different peaks with different retention times indicating that they produced different compounds. When a carbon source was not included in the fermentation media, the antimicrobial production was substantially reduced to almost eight times. Carbon source in the fermentation medium plays a vital role in the production of antimicrobial substances. Yeast extract and fructose as nitrogen and carbon sources in the fermentation medium produced maximum antimicrobial activity.

Keywords: antimicrobial metabolites, carbon sources, fermentation, media formulation

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 started 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; Wang *et al*. 2011). For *Xenorhabdus* sp. D43 the carbon sources that enhanced the antimicrobial substance production by was maltose and glycerol respectively (Yang *et al.* 2006) but *X. nematophila* BJ it was glucose (Yang *et al*. 2001). 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).

The entomopathogenic nematode/bacterium (EPN/EPB, Steinernema/*Xenorhabdus* and *Heterorhabditis/Photorhabdus*) symbiotic associations (Goodrich-Blair and Clarke 2007) are potential tools for biological control of insect pests (Gaugler 2002; Ffrench-Constant *et al.* 2007) and microbial pathogens (Böszörményi *et al.* 2009) of agricultural importance. EPN infested insect cadaver does not purify. This is being achieved by the production of a number of bioactive compounds by the bacterium which can

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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, 1995b). Thus Insect-nematode-bacterium tripartite associations such as those involving *Xenorhabdus* species provide attractive systems for both discoveries of new natural products, identification of novel compounds involved in inter kingdom signaling and antibiotics (Park *et al.* 2009). These metabolites not only have diverse chemical structures, but also have a wide range of bioactivities of medicinal and agricultural interest, such as antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastic and antiviral (Webster *et al.* 2002).

We report here the effect of six carbon sources plus yeast extract on antimicrobial metabolite production by an entomopathogenic bacterium isolated from rhabditid nematode to produce diverse compounds in different media.

MATERIALS AND METHODS

All chemicals used for extraction were of AR grade (Merck, Mumbai, India). Nutrient agar, Mueller Hinton Agar (MHA), nutrient broth, potato dextrose agar, potato dextrose broth and yeast extract were purchased from the Himedia Laboratories Ltd., Mumbai, India. The carbon sources used are fructose, maltose, dextrose, mannitol, sucrose and lactose were purchased from the SRL Laboratories Ltd., 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 *E. coli* MTCC 2622; agriculturally important fungi *Pencillium expansum* (local isolate) and medically important fungi *Candida albicans* MTCC 277. Nutrient agar was used for subculturing the bacteria and potato dextrose agar slants for subculturing the fungi.

Antibiotics-producing bacteria

The antimicrobial-substance producing bacterium was isolated from an entomopathogenic, *Rhabditis (Oscheius)* species 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 (Deepa *et al*. 2011). The 16S rDNA sequence was deposited with NCBI (accession number AHQ200404). The bacterium has been deposited with IMTECH, Chandigarh (accession number is MTCC 5234).

Fermentation media

The bacterial isolate was inoculated into liquid medium. The liquid media was prepared with different carbon sources (fructose, maltose, dextrose, mannitol, sucrose and lactose) as the first factor nutrient and yeast extract as the second nutrient source. The liquid media were 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, Remi CS 24, Mumbai, India (150 rpm) at 30°C in darkness for 24–48 h. When the optical density (Systronics double beam spectrophotometer 2201 UV-VIS) of the culture at 600 nm was approx 1.7, the bacterial cultures were transferred into 400 ml of sterile medium and incubated in a gyrorotatory shaker (150 rpm) at 30°C in darkness for 96 h. The culture media were then centrifuged (10,000 rpm, 20 min, 4°C) followed by filtration through a 0.45 µM syringe filter (Millipore), to obtain a 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 three times. The ethyl acetate extracts were combined, dried over anhydrous sodium sulphate, and concentrated using a rotary flash evaporator (Buchi Rotavapor R-210) 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 and incubated at 37°C for 18 h and were suspended in saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 McFarland standards (10^6 CFU/ml). The suspension was used to inoculate on MHA plates. Sterile paper discs (6.0 mm diameter, Whattman antibiotic assay disc) impregnated with 1 mg/ml 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 h to allow the diffusion of the crude extracts and then inverted and incubated for 18-24 h at 37°C. The diameter of inhibition zone was measured in mm. Ciprofloxacin (5 µg/ml) (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 PDA medium. Paper disc (6 mm) was placed on the surface of the seeded PDA plates and 25 µg/ml of each crude extract was added and air dried in a 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 (LC-10AT liquid chromatograph, Shimadzu, Japan). Sample (20 µL) was injected into a C18 column (250 mm × 4.6 mm × 5 mm). The flow rate was 1 ml/min 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). Treatment effect was tested by ANOVA and the means compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level.

RESULTS

Yield of crude extract

Six different carbon and yeast extract at a concentration of 1% were used in the present study to find out the ideal carbon sources having enhanced yield and antimicrobial activity. A total of 7 different combinations were used in the present study. There was a high degree of variation in the yield of crude extract when the carbon sources in the fermentation medium changes (**Fig. 1**). The yield was the highest in yeast extract plus maltose (836 ± 4.58 mg/L) followed by yeast extract and dextrose. The lowest crude yield extract was recorded for malt extract. 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. 1**).

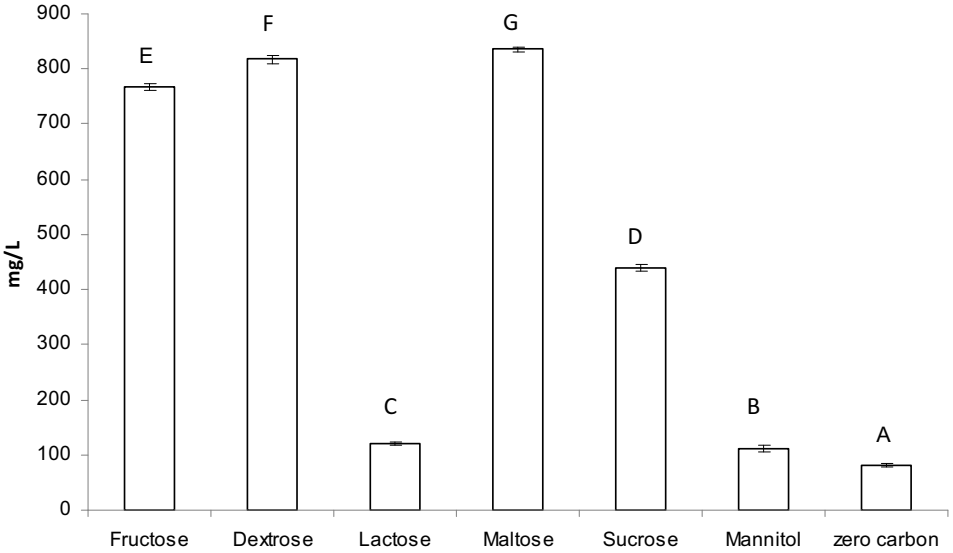

Fig. 1 Yield of crude extract of yeast extract plus six different carbon sources. Values represents mean of three replications. Different letters indicate significant differences between means at $P < 0.05$ according to DMRT.

Antimicrobial activity of crude extract

There was a high degree of variation in the level of antimicrobial activity against the test microbes when the different carbon sources were added in the fermentation medium (**Table 1**). 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) (**Table 1; Fig. 2**). Yeast extract and maltose also recorded a similar pattern of activity against the test microorganisms. Lactose, sucrose and mannitol as carbon source in general produced less antimicrobial activity. Antimicrobial activity was considerably decreased when nitrogen sources alone were used in the fermentation media (**Fig. 2**). 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.

Table 1 Antimicrobial activity of crude extract measured as a zone of inhibition (diameter in mm).

Carbon sources	*B. subtilis*	*E. coli*	*C. albicans*	*P. expansum*
Fructose	42.33 A	42.00 A	26.00 C	46.5 D
Glucose	14.67 C	13.67 A	12.33 A	11.33 A
Lactose	22.00 A	24.00 B	14.67 C	14.67 D
Maltose	41.00 D	35.00 A	26.67 B	42.33 C
Sucrose	32.00 A	19.00 B	11.33 C	9.33 D
Mannitol	23.66 A	21.33 A	13.67 B	12.67 B
Zero carbon	24.00 A	11.67 B	10.33 B	5.33 C

Average of three replications. Means in a column with the same letter(s) do not significantly different at $P < 0.05$ according to DMRT.

HPLC analysis

The HPLC data of crude ethyl acetate extract are given in **Fig. 3A-F**. In HPLC analysis yeast extract and fructose recorded 59 compounds and retention time (Rt) ranges from 0.46 to 33.51 min (**Fig. 3**). Even though the combination of yeast extract with fructose and maltose recorded almost similar bioactivity, yeast extract plus maltose recorded only 42 compounds (**Fig. 3**). 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. The retention time of the peaks with different combinations was quite different in each combination indicating the production of different molecules. Using still different carbon and nitrogen sources along with different combination of minerals and many other constituents may produce more compounds.

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 by *Streptomyces* species (Rizk and Metwally 2007), lactose (Petersen *et al.* 1994), sucrose by a new *Streptomycete* (Charkrabarti and Chandra 1982), fructose by *Streptomyces thermoviolaceou* (James and Edwards 1988), starch (Kotake *et al.* 1992) and glycerol by *Streptomyces hygroscopicus* (Bhattacharyya *et al.* 1998) have been reported to be suitable for production of secondary metabolites. Galactose and glucose strongly enhanced the antimicrobial activity of *Corynebacterium kutscheri* and *C. xerosis* respectively, while ribose and lactose repressed their activity (Gebreel *et al*. 2008). The choice of carbon sources greatly influenced secondary metabolism and therefore antibiotic production (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 culti-

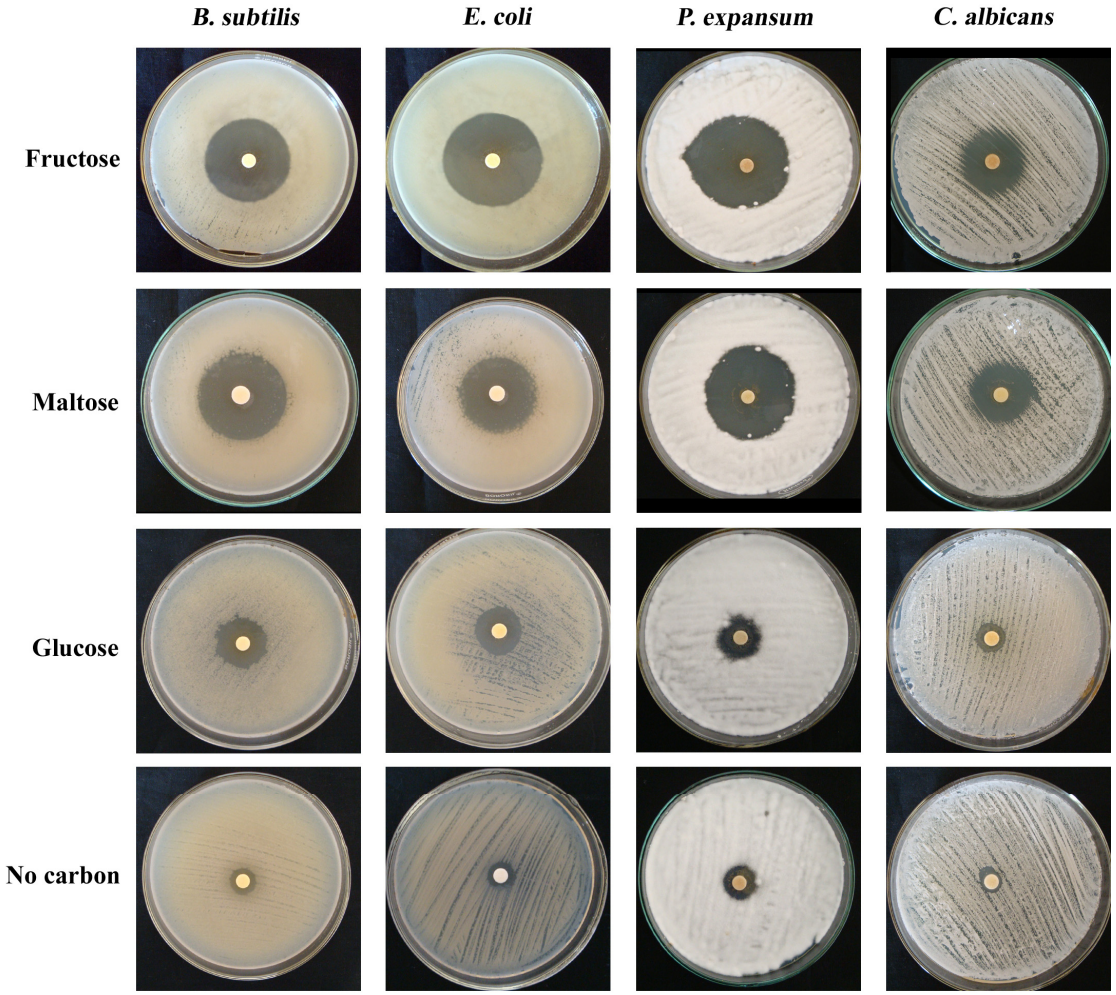

Fig. 2 Antimicrobial activity of crude extract from yeast extract plus sugars.

vation 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). In particular, in the field of antibiotics, much effort was directed toward optimizing production rates and directing the product spectrum. 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 bacteria and other microorganisms, glucose, usually an excellent carbon source for growth interferes with the formation of many antibiotics (Demain 1989). In the present study also dextrose recorded significantly higher yield of crude extract with low antimicrobial activity. 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 in *Streptomyces hygroscopicus*.

Antibiotic production by EPB differs qualitatively and quantitatively in the strain types and species (Fang *et al.* 2010). Besides, growth medium and fermentation conditions also play very important roles. Cell growth and the accumulation of metabolic products are strongly influenced by growth medium and fermentation conditions such as carbon sources, nitrogen sources, inorganic salts, pH, temperature, agitation and oxygen availability (Fang *et al.* 2010). EPB cultivated in 1% peptone water showed no antimicrobial activity, however, other media including yeast extract broth and its modifications (Sundar and Chang 1993), Luria–Bertani broth (Sundar and Chang 1993) and TSB (Ji *et al.* 2004) have been used successfully for antibiotic production by *Xenorhabdus* sp. and *Photorhabdus* sp.. Similarly in our study yeast extract plus fructose recoreded high antimicrobial activity. Limiting the supply of nutrient not only is an effective means to restrict cell growth but also has specific metabolic and regulatory effects (Doull and Vining 1990). Therefore, to achieve high product yields, one prerequisite is to design a proper production medium. There is usually a relationship between the growth medium and the biosynthesis of antibiotics (Elibol 2004). An ideal carbon and nitrogen source is required for the biosynthesis of antimicrobial compounds. In our study fructose and maltose play a significant role in the antimicrobial compound production irrespective of the nitrogen source (yeast extract).

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). It was noted by Sanchez and Demain (2002) that ammonium salts did not favour biosynthesis of novobiocin, actinomycin, neomycin, kanamycin and others, but for rapamycin ammonium sulfate was the best nitrogen source (Lee *et al.* 1997). 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

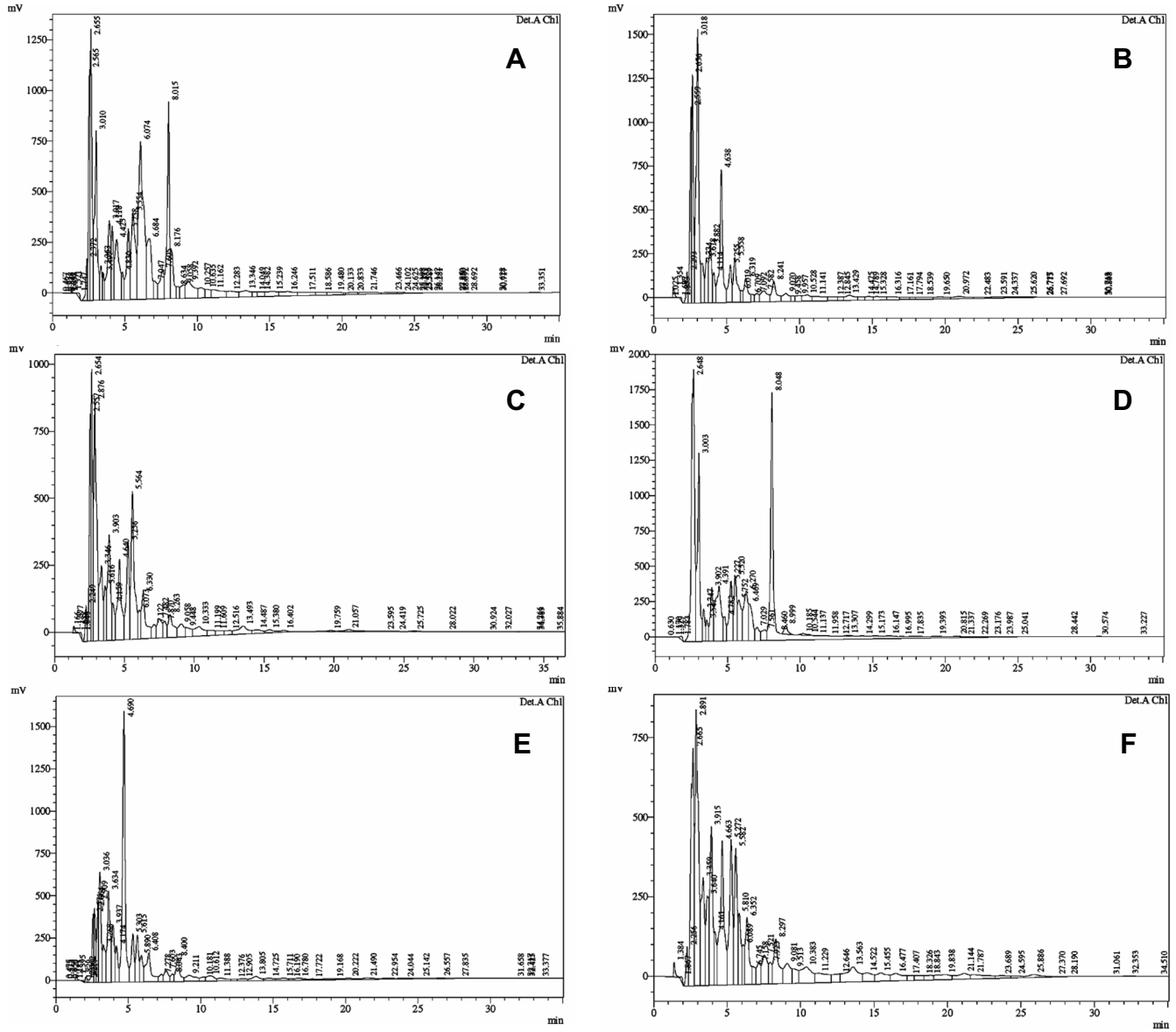

Fig. 3 HPLC chromatogram of the crude extract of yeast extract and carbon sources on reversed-phase C18 column (LC-20AD). Samples of 20 µL were injected to a column (250 mm ×4.6 mm × 5 mm), eluted with methanol:water (50:50). (**A**) Fructose, (**B**) Glucose, (**C**) Lactose, (**D**) Maltose, (**E**) Sucrose, (**F**) Mannitol.

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

Regulation by the carbon source depends on the rapid utilization of the preferred carbon source. Different mechanisms have been described the negative carbon catabolite effects on antibiotic production. These mechanisms show important differences depending on the microbe being considered. For a desired antibiotic to become a commercial reality, the genetic improvement in its production is a necessary step before getting the required titers for industrial production. Both strain improvement and nutritional modification will preclude the scaling up into the pilot scale by biochemical engineers and later, into factory size fermentors. 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.

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