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Original Article

INFLUENCE OF SIX NITROGEN SOURCES WITH FRUCTOSE ON ANTIMICROBIAL METABOLITE PRODUCTION BY BACTERIUM ASSOCIATED WITH ENTOMOPATHOGENIC NEMATODE

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ABSTRACT

Objective: 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 nitrogen sources in combination with fructose on the production of antimicrobial substances by *Bacillus* sp.

Methods: Bacterial fermentation was carried out using fructose as carbon source and six nitrogen sources (meat peptone, beef extract, yeast extract, tryptone, meat infusion extract and malt extract). Antibacterial activity of the crude extract was studied using disc diffusion assay. The component present in crude extract was studied using analytical HPLC.

Results: The yield of crude antimicrobial substances and antimicrobial activity against the test microorganism also differed significantly when the nitrogen sources in the fermentation media were changed. The highest yield was recorded for beef extract plus fructose (921 mg/L). The antimicrobial activity was significantly higher in yeast extract plus fructose [*Penicillium expansum* (46.5 ± 2.12 mm) and *Escherichia coli* (42.00 mm)] than yeast extract plus other carbon sources 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.

Conclusion: 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, EPN, Nitrogen sources, Fructose.

INTRODUCTION

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 [1]. 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 [2]. 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 [3].

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 [4]. 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 [5].

It is well known that 30-40% of the production cost of antibiotics is taken up by the cost of growth medium [6]. 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 [7]. 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 [8]. 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 [9].

MATERIALS 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 source used is fructose was 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

bacterium 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 fructose 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 °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 °C in darkness for 96 hours. The culture media were then centrifuged (10,000 rpm, 20 min, 4 °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 [10]. 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, Whattman 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 h 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 [11]. 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 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 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

Yield of crude extract

Six different nitrogen and fructose at a concentration of 1% were used in the present study to find out the ideal nitrogen sources having enhanced yield and antimicrobial activity. A total of 6 different combinations were used in the present study. There was a high degree of variation in the yield of crude extract when the nitrogen sources in the fermentation medium changes (Fig. 1). The yield was the highest in beef extract plus fructose (921 ± 4.58 mg/L) followed by yeast extract and fructose. 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).

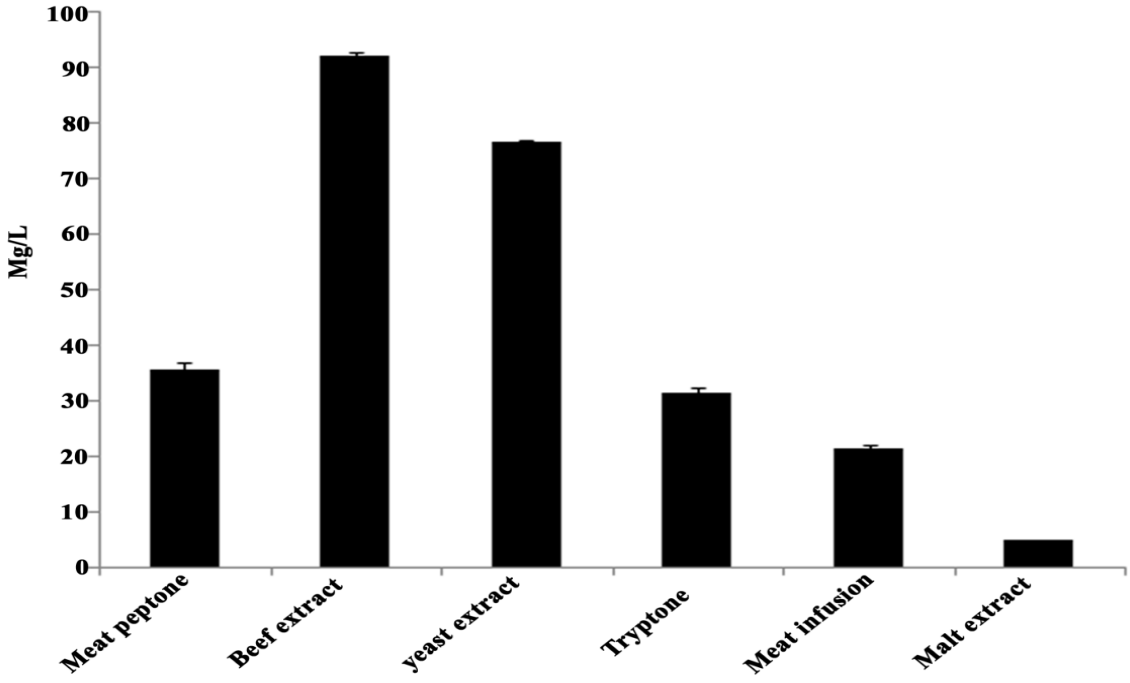


Fig. 1: Yield of crude extract of fructose plus six different nitrogen sources. Values represents mean of three replications

Table 1: Antimicrobial activity of crude extract

	Zone of inhibition Dia. in mm Fructose			
Nitrogen sources	***B. subtilis***	***E. coli***	***C. albicans***	***P. expansum***
Meat peptone	23±0.67^{dBC}	21±0.67^{dB}	20.00±0deB	14.00±0^{cA}
Beef extract	38.00±0^{fD}	31±1.67^{eD}	21.00±1eB	31±1.33^{gC}
Yeast extract	42±0.33^{dE}	42.00±1^{eE}	26.00±1dC	46±0.5^{fD}
Tryptone	20±1.66^{dB}	28±0.67^{eC}	12.00±0bcA	12±0.33^{bcA}
Meat infusion	25±0.67^{eC}	21±1.67^{cB}	20.00±0fB	13.00±0^{bA}
Malt extract	10.00±0^{aA}	8.00±0^{bA}	19±1.12cB	19±1.67^{eB}

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

Meat peptone

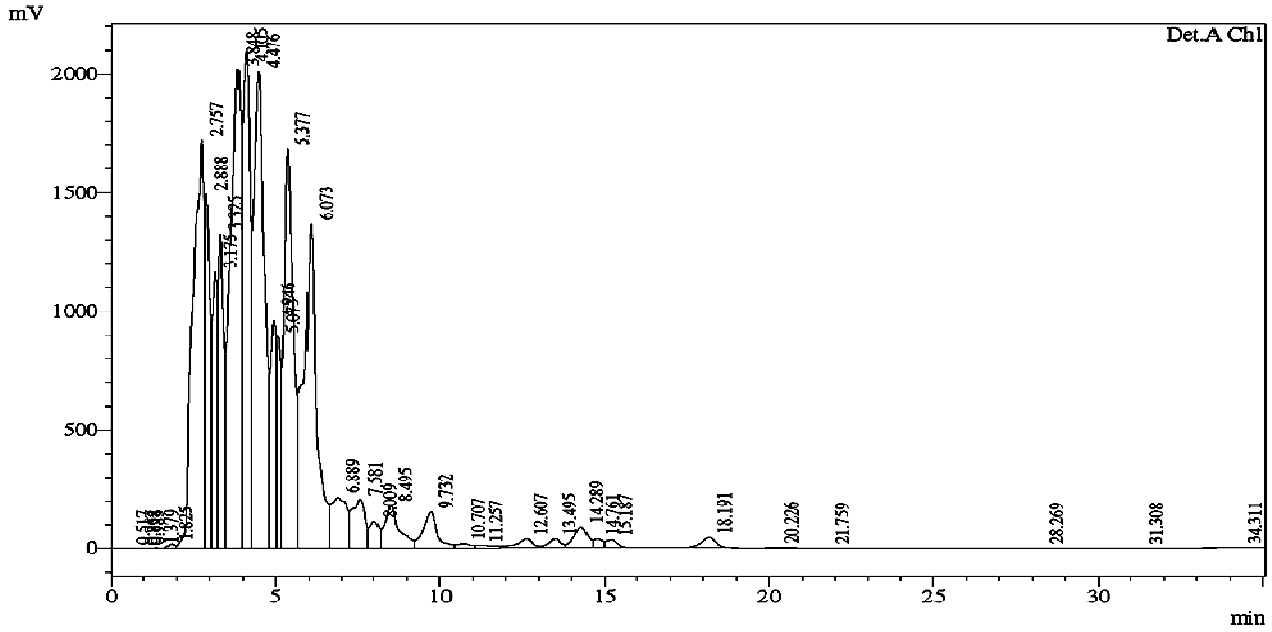
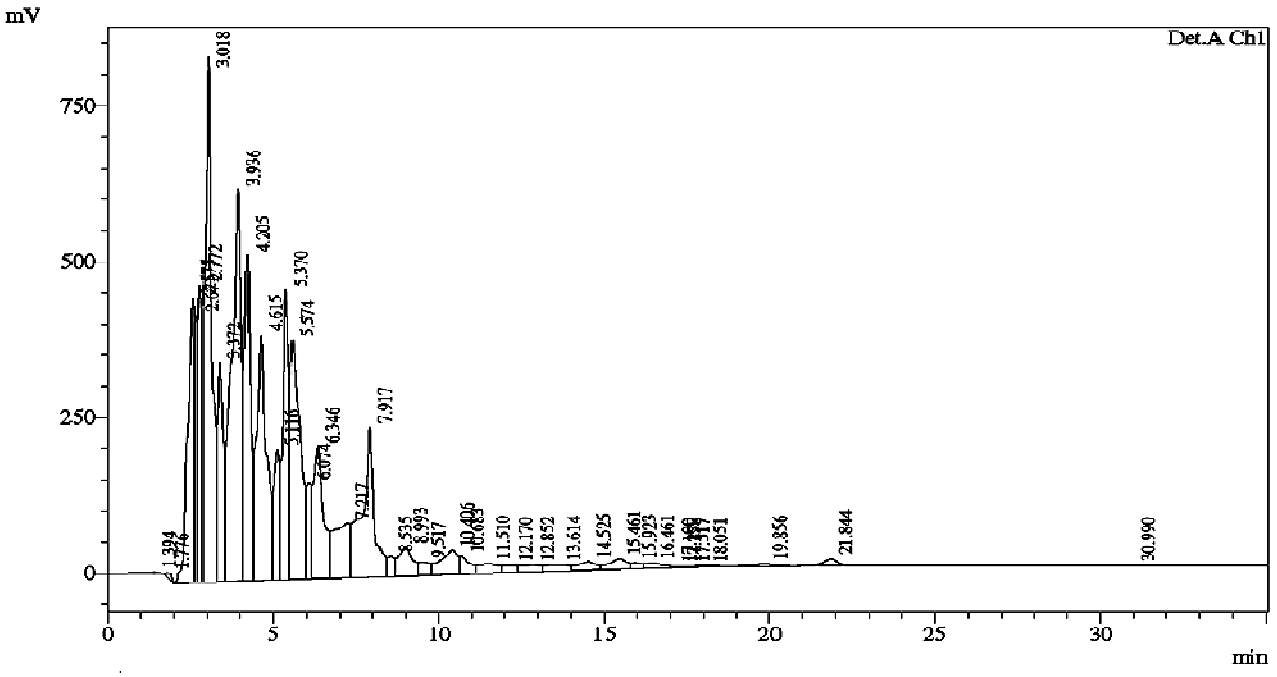
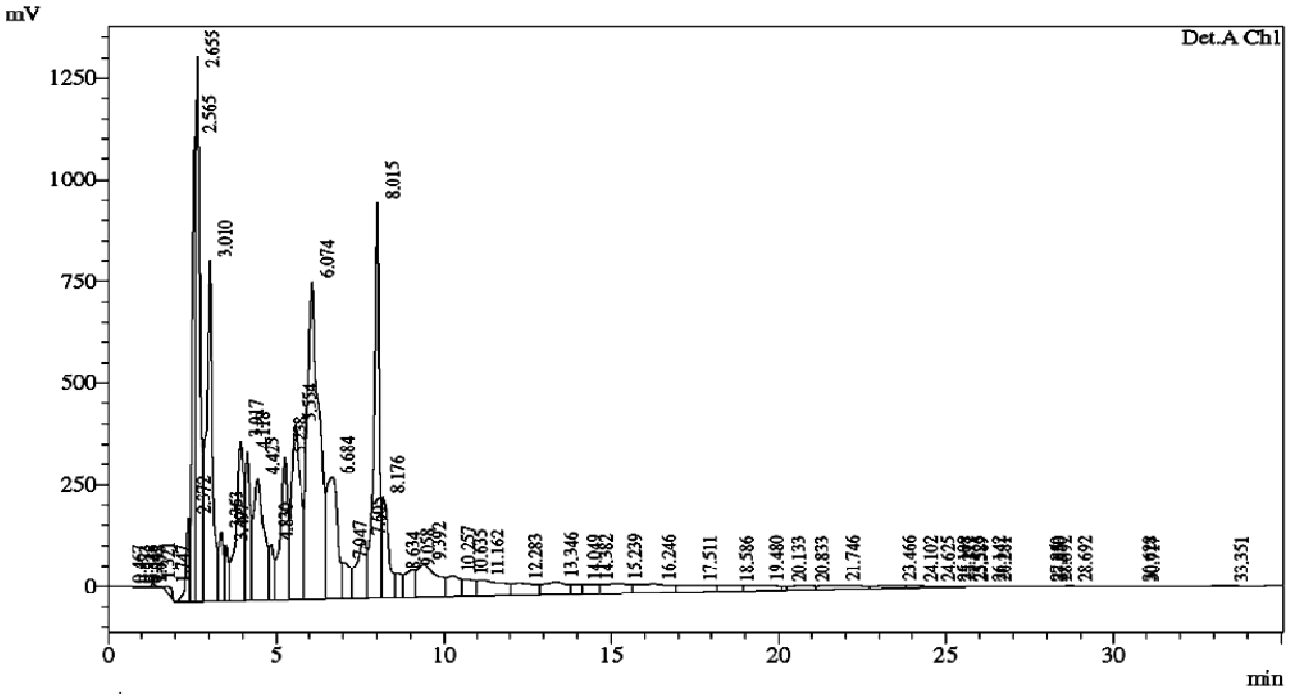


Fig. 2: HPLC chromatogram of the crude extract of six nitrogen source and fructose.

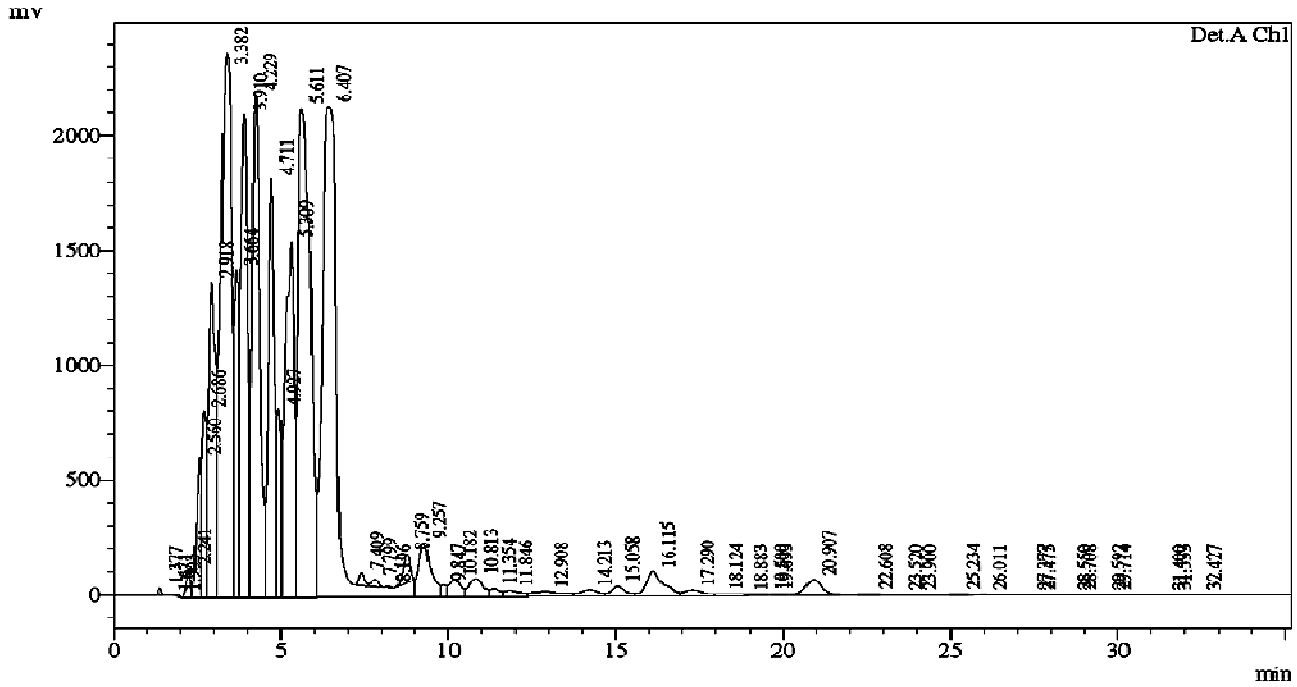
Beef extract



Yeast extract



Tryptone



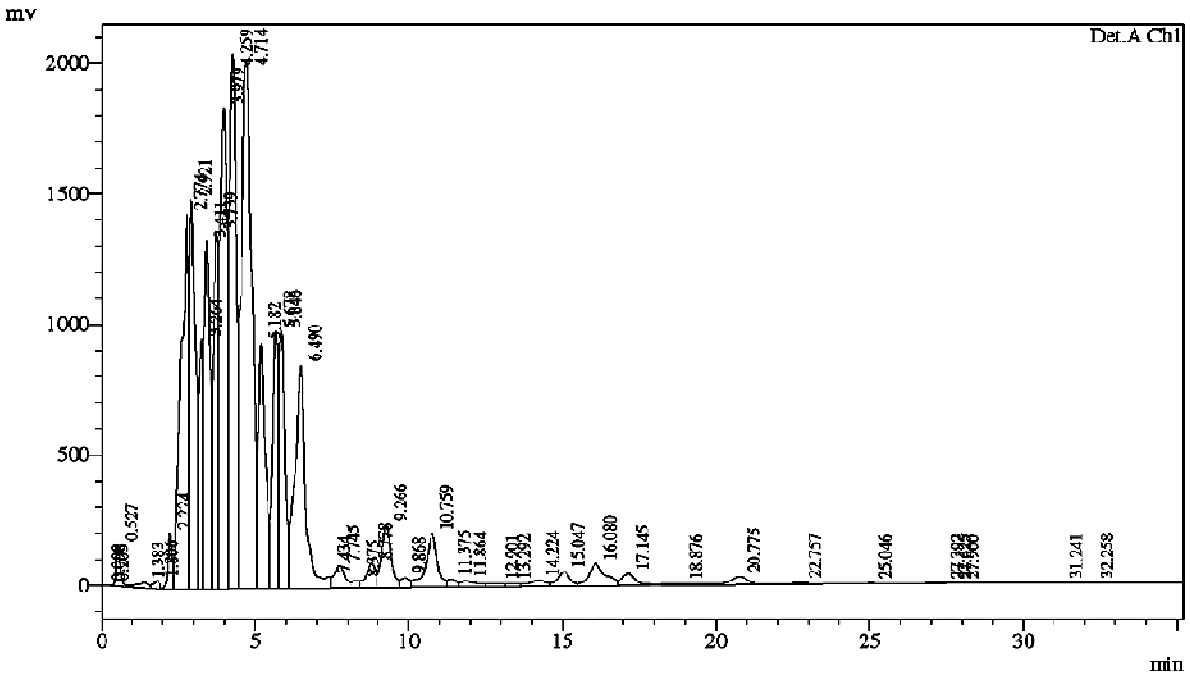
Antimicrobial activity of crude extract

There was a high degree of variation in the level of antimicrobial activity against the test microbes when the different nitrogen 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). Malt extract as nitrogen source in general produced less antimicrobial activity. The result of the present study clearly indicated that an ideal carbon and nitrogen sources are needed for better antimicrobial activity and best nitrogen source in our study was yeast extract.

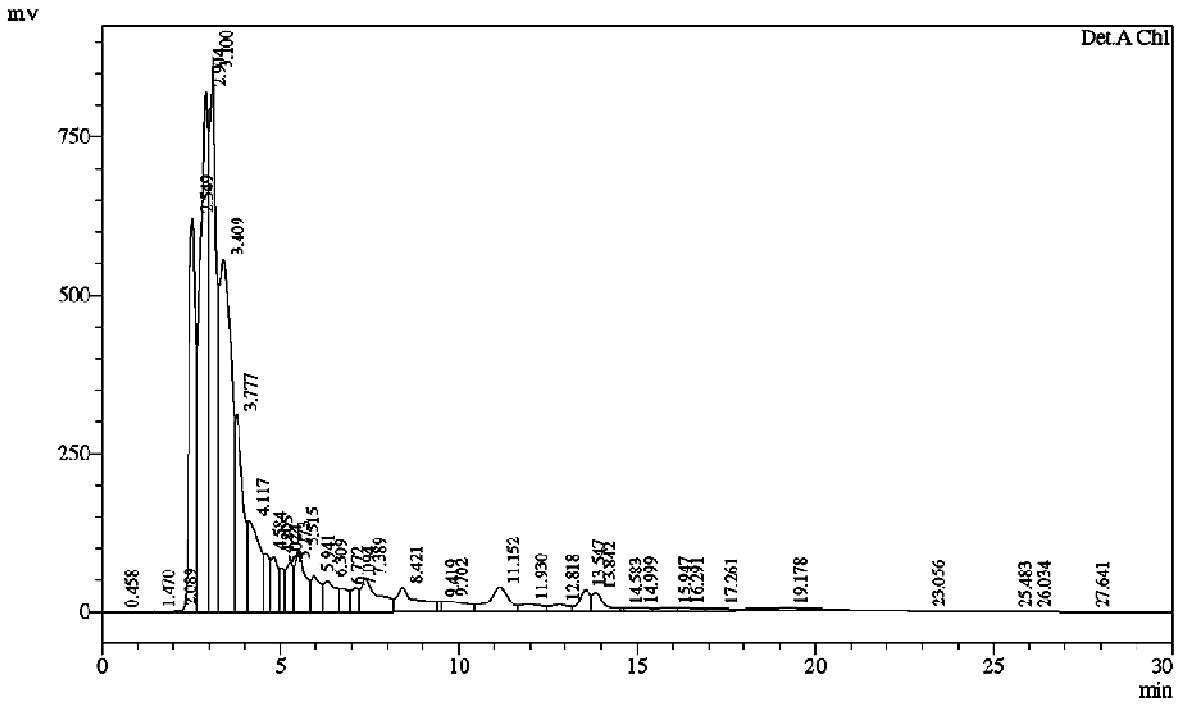
HPLC analysis

The HPLC data of crude ethyl acetate extract are given in Fig. 2. In HPLC analysis yeast extract and fructose recorded 59 compounds and retention time (Rt) ranges from 0.46 to 33.51 min (Fig. 2), beef extract plus fructose recorded only 42 compounds (Fig. 2). 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.

Meat infusion



Malt extract



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 [12]. 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 [12]. Formation of antibiotics is also regulated by nutrients (such as nitrogen, phosphorous and carbon source), metals, growth rate, feedback control and enzyme inactivation [13]. 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 [14, 15] .

The nature of the nitrogen source used has a notable effect on the production of the antimicrobial metabolite in *A. terreus.* High

nitrogen levels have been noted to repress idiophase production of antibiotics [16]. Control of ammonia concentration during the mid-cycle was found to be important in the optimization of idiophase secondary metabolite production [17], though this may reflect the role of nitrogen in growth promotion. The use of certain amino acids as a nitrogen source can inhibit good synthesis of secondary metabolites [18].

Antibiotic production by EPB differs qualitatively and quantitatively in the strain types and species [19]. 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 [19]. EPB cultivated in 1% peptone water showed no antimicrobial activity, however, other media including yeast extract broth and its modifications [20], Luria–Bertani broth [20] and TSB [21] have been used successfully for antibiotic production by *Xenorhabdus* sp. and *Photorhabdus* sp.. Similarly in our study yeast extract plus fructose recorded 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 [22]. 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[23]. 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 [24]. It was noted by Sanchez and Demain [13] that ammonium salts did not favor biosynthesis of novobiocin, actinomycin, neomycin, kanamycin and others, but for rapamycin ammonium sulfate was the best nitrogen source [25]. 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 22] .

CONCLUSIONS

It was demonstrated in this study that different nitrogen sources had remarkably distinct effects on cell growth and antibiotic activity of *Bacillus sp.* in shaking flasks. In addition to nitrogen sources, other parameters such as dissolved oxygen (including agitation speed and aeration rate) and temperature may also be changed in the two-stage process to optimize cell growth and antibiotic production, respectively. However, this hypothesis remains to be verified further.

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