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Optimization of media and temperature for antimicrobial activity of *Enterobacter* sp. associated with entomopathogenic nematode *Rhabditid* sp.

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ABSTRACT: An entomopathogenic bacterium isolated from the nematode, *Rhabditis* (*Oscheius*) sp. was found to produce secondary metabolites with antimicrobial activity. The bacterium isolated from the entomopathogenic nematode was identified as *Enterobacter* sp. by using biochemical and 16S rDNA sequence analysis. Media for the production of the bioactive metabolites were standardized with six carbon sources viz. glycerol, maltose, fructose, glucose, sucrose and lactose, and four nitrogen sources viz. tryptone, yeast extract, beef extract and peptone. Antimicrobial activity was found highest for culture filtrate solvent extract (CFSE) obtained from tryptone plus glycerol (T+G) combination. Addition of peptone to the media, irrespective of carbon sources, had the least antimicrobial activity. Fermentation with tryptone plus glycerol medium when carried out at temperature ranging from 25 to 40 ºC, the highest antimicrobial activity was observed at 37 ºC.

KEYWORDS: *Rhabditis (Oscheius)* sp., antimicrobial, entomopathogenic bacteria, *Enterobacter* sp.,

INTRODUCTION

The bacteria *Xenorhabdus* and *Photorhabdus* are symbiotically associated with nematodes belonging to the families Steinernematidae and Heterorhabditidae, respectively Poinar (1990). Virulence of entomopathogenic nematodes (EPN) to insects is attributed due to its symbiotic bacteria associated with EPN Babic *et al.*, (2000). The importance of entomopathogenic bacteria (EPB) as a source of antibacterial and antifungal molecules has been studied in detail (Webster *et al*., 2002; Bode *et al*., 2009).

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Rhabditis (*Oscheius*) spp. isolated from different agroclimatic zones of Kerala resemble EPN and was found to be effective for the control of arecanut spindle bug in the field (Mohandas *et al*., 2004). These were found to kill a number of important insect pests within 24-72 h in laboratory conditions. Red ants and termites have been found to be effectively killed by the EPN in the field. They could also control mealy bugs by killing associated ant colonies (Mohandas *et al.*, 2007). Moreover the EPB associated with *R*. (*Oscheius*) sp. represent an important source of bioactive molecules with antimicrobial activity (Kumar *et al*., 2014). Media such as yeast extract broth and its modifications (Akhrust *et al.*, 1982; Sundar and Chang, 1993), Luria.Bertani broth (LB) (Sundar and Chang, 1993), sea water (Paul *et al*., 1981) and Tryptic Soya Broth (TSB) (Li *et al*., 1997; Ji *et al*., 2004) are used successfully for the production of antimicrobial metabolite from EPB. The production of antimicrobial metabolite by EPB is strongly influenced by culture medium and fermentation conditions such as pH, temperature, agitation and oxygen availability (Fang *et al*., 2010). A high degree of variation in the level of antimicrobial activity of *Bacillus* sp. associated with *Rhabditis* (*Oscheius*) sp. was reported by changing carbon and nitrogen sources in the fermentation media (Kumar *et al*., 2012). The present study was conducted to optimise different culture media for the maximization of antimicrobial activity of *Enterobacter* sp.

MATERIALS AND METHODS

Isolation and identification of Entomopathogenic bacterium: The bacterium was isolated from EPN *Rhabditis* (*Oscheius*) sp infected cadavers of *Galleria mellonella* 48-hour post infection according to the method as described by (Woodring and Kaya, 1988). The isolated bacterium was subcultured monthly and maintained in the laboratory.

Identification of the bacterium was made based on the morphological, biochemical and 16S rRNA gene sequences. Total genomic DNA of the bacteria was extracted as per the approved protocol (Reinhardt *et al*., 2008). Bacterial 16S rDNA was amplified by using bacterial universal primers: forward primer fD1 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer RP2 5'CGGCTACCTTGTTACGACTT3' (Weisburg et al. 1991). The 16S rDNA gene sequences were obtained by sequencing the PCR product. The gene sequences of the bacterium obtained were aligned with Clustal alignment programme of MEGA software (Tamura *et al.,* 2011) and the nucleotide sequences were compared with those in the NCBI database using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

Fermentation media preparation: The fermentation media (g L^{-1}) were prepared with carbon source 10.0, nitrogen source 10.0, K_2HPO_4 2.0, KH_2PO_4 2.0, $MgSO_4$ 1.0, NaCl 1.0 and Na_2SO_4 1.0. Bacteria were inoculated into the liquid medium, that was prepared with different carbon sources such as glycerol, maltose, fructose, dextrose, sucrose, lactose, and nitrogen sources such as tryptone, yeast extract, beef extract, peptone, each at a level of 1% (W/V). pH of the media was adjusted to 7.0 before autoclaving. Tryptone plus glycerol medium (T+G) was fermented at different temperature regime (25, 30, 35, 37 and 40 ºC), and with L-Proline at different concentrations (25, 50, 75 and 100 mM) at varying time interval (24, 48, 72, 96 and 120 h).

Preparation of culture filtrate: The medium was taken in separate 250 mL Erlenmeyer flasks and was inoculated with a loop full of the bacterial culture. The media were incubated in a gyrorotatory shaker with 150 rpm at 30 ºC in dark for 24 h. Optical density of the culture was determined by using a spectrophotometer at 600 nm and when it reached 1.7, these cultures were transferred into 400 mL fresh sterile medium and further incubated in a gyrorotatory shaker with the same condition for 24, 48, 72, 96 and 120 h. The culture media were centrifuged at 10,000 rpm for 20 min at 4 ºC and passed through a 0.45 µM filter to obtain cell free culture filtrate.

Preparation of solvent and aqueous fraction of culture filtrate: The cell free culture filtrate, 500 mL, was neutralized with 1 N HCl and extracted with an equal volume of 500 mL ethyl acetate. The extraction was repeated twice, the culture filtrate aqueous and solvent fractions were separated by using a separating funnel. The culture filtrate solvent extract (CFSE) was concentrated using a rotary flash evaporator at 40 ºC, and the metabolite obtained was weighed, reconstituted in methanol and used for assay of antimicrobial activity.

Antimicrobial assay: Antibacterial and antifungal activity of the CFSE were studied against agriculturally important fungi and medically important bacteria and fungi, by the agar well diffusion assay as described in Clinical and Laboratory Standards Institute (CLSI), 2008. Test pathogens which includes Gram positive bacteria, *Bacillus subtilis,* MTCC 2756*; Staphylococcus aureus,* MTCC 902, and Gram negative bacteria *Escherichia coli,* MTCC 2622, and *Pseudomonas aeruginosa,* MTCC 264 were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Commercial antibiotics ceftazidime 30 $\mu g.mL^{-1}$ and ciprofloxacin 5 $\mu g.mL^{-1}$ were used as positive reference standard. Agriculturally important fungi like *Fusarium oxysporum,* MTCC 284, *Rhizoctonia solani,* MTCC 4634, *Penicillium expansum,* MTCC 2006, and medically important fungi *Aspergillus flavus,* MTCC 183, *Candida albicans,* MTCC 277 were also obtained from IMTECH. Amphotericin was used as control for *C. albicans,* whereas Carbendazin 100 $\mu g.mL^{-1}$ was used for the remaining four fungi. Methanol was kept as control along with the test samples for antibacterial and antifungal activity. Diameter of the inhibition zones was measured.

Statistical analysis: Data were analysed using SPSS (Version 17.0; SPSS, Inc., Chicago, IL, USA). Means of the samples were compared using univariate ANOVA with zone of inhibition as dependent variable. Statistical significance was defined as $p< 0.05$.

RESULTS AND DISCUSSION

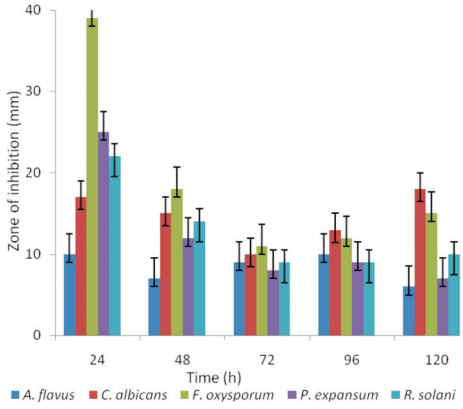
Identification of Entomopathogenic bacterium (EPB): Colonies of bacteria observed were irregular with undulate margins, devoid of pigmentation and measured 2.6 to 0.69 µm. It was Gram negative, rod shaped and motile. Carbohydrate fermentation test revealed that the bacterium could utilize glycerol, maltose, glucose, fructose, mannitol, sucrose, starch as carbon source. The gene sequencing confirmed the bactierium as *Enterobacter* sp. and the sequence was deposited in the NCBI Gen Bank with the accession number JX470959.

Antimicrobial activity of extract: Antimicrobial activity of the CFSE obtained from T+G was significantly higher followed by tryptone plus maltose (T+M) at 30 ºC for 24 h (Fig. 1-2), and the highest antifungal activity was observed against *Fusarium oxysporum.* In both cases the zone of inhibition for CFSE from the medium with T+G was, 39.00±1.00, 25.33±1.52, mm respectively against *F. oxysporum* and *P. expansum*. The CFSE obtained from yeast extract plus glycerol and yeast extract plus maltose media also recorded highest antimycotic activity against *F. oxysporum* and *P. expansum* (Fig. 3-4). The antibacterial activity was recorded highest in the CFSE obtained from T+G and T+M against *E. coli* and *B. subtilis* respectively (Fig. 6-7). Antimicrobial activity of CFSE obtained from medium with peptone as nitrogen source (irrespective of its carbon sources) was insignificant against the test organisms (Table 1 and 2).

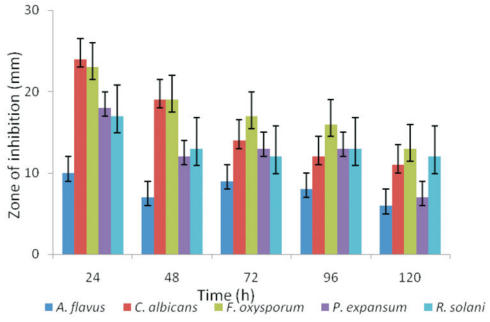
Influence of temperature and L-Proline on antimicrobial activity: The effect of temperature on the antimicrobial activity of CFSE obtained from (T+G) medium at 25, 30, 35, 37 and 40 ºC revealed that the maximum antimycotic activity was at 37 ºC (Table 3). A high degree of variation in the level of antimicrobial activity against the test microbes were observed when L-Proline was added in the media at different concentrations. Of the different concentrations of L-Proline added, the highest antimicrobial activity was observed at 75 mM (Fig. 5).

The present study was conducted with the aim to optimise media and temperature to get maximum antimicrobial activity of *Enterobacter* sp. associated with EPN *Rhabditis (Oscheius)* sp. *Enterobacter* sp. are very common in insects and normally found in soil and water (Bucher 1981). Previous studies also reported isolation of three insect pathogenic bacteria *Enterobacter aerogenes*, *E. agglomerans* and *E. cancerogenus* against larvae of *Oberea linearis* (Bahar and Demirbag 2007). The insecticidal activity of *Enterobacter* sp., *E. aerogenes* against larvae of *Melolontha melolontha* and *Agrotis segetum* with 20, 60 % mortality was reported by (Sezen *et al.*, 2007; Sevim *et al.*, 2010).

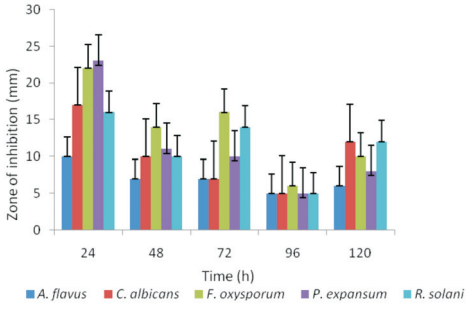
Among the different carbon sources tested, glycerol proved to be the best for antimicrobial activity and that was followed by maltose, fructose and glucose. Variations in the fermentation conditions often result in an alteration in antibiotic production. The carbon source needed for maximal yield of the antibiotic production also seems to be different among bacterial strains. The choice of carbon sources greatly influenced secondary metabolism and antibiotic production (El-Banna 2006; Martin and Demain 1978). Glycerol is known to be an important medium component for the production of antifungal metabolites from microorganisms Fukuda *et al.,* (2005). It was also reported that maltose and glycerol had the strongest effect on the antibiotic activity of *Xenorhabdus* sp. D43 Yang *et al.*, (2006). In the current investigation tryptone was identified as the best nitrogen source for antimicrobial activity, but it was significantly less when peptone was replaced as nitrogen source. This indicates that the source of nitrogen has a pivotal role in the production of antimicrobial activity. Carbon and nitrogen sources are the important nutritional components of the medium to influence the antibiotic activity of *X. nematophila* (Yang *et al*., 2001; Wang *et al*., 2008). The *Enterobacter* sp. showed maximum antimicrobial activity at 24 h followed by 48 and 72 h which is followed



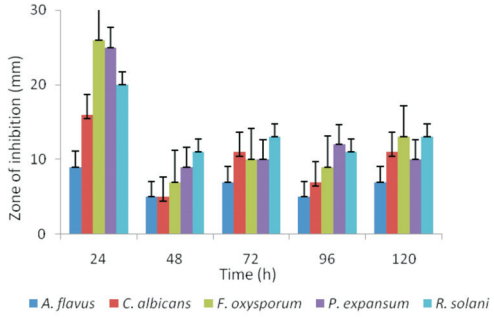
1. Tryptone plus glycerol



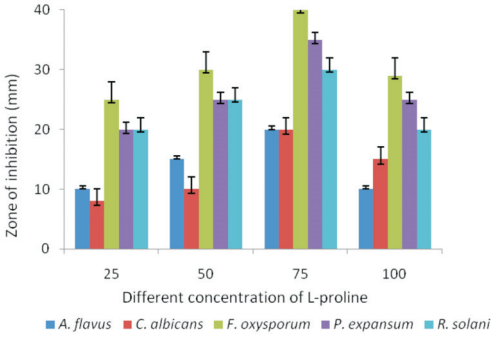
2. Tryptone plus maltose



3. Yeast extract plus glycerol

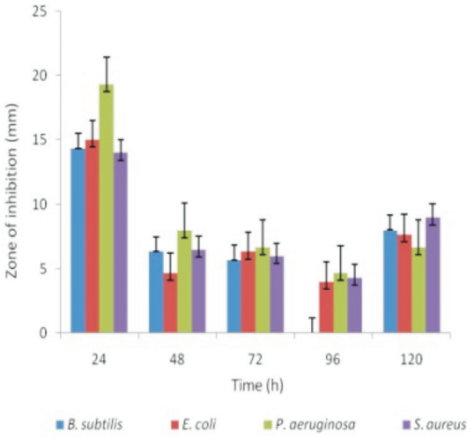


4. Yeast extract plus maltose

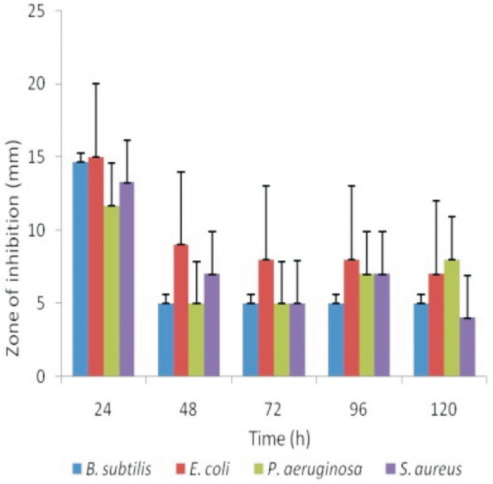


5. Tryptone plus glycerol with L-Proline

Figs (1 – 5) Antifungal activity of culture filtrate solvent extract of *Enterobacter* sp. obtained from different media



6. Tryptone plus glycerol



7. Tryptone plus maltose

Figs (6-7) Antibacterial activity of culture filtrate solvent extract of *Enterobacter* sp. with different media

by a stationary phase. The antimicrobial metabolite production was detected even after 72 h in the late exponential and stationary phase upto 120 h which may be due to the influence of different carbon sources on secondary metabolite production. Different carbon sources, like dextrose (Rizk and Metwally 2007), lactose (Petersen *et al.,* 1994), sucrose (Charkrabarti and Chandra 1982), fructose (James and Edwards 1988) and starch (Kotake *et al*., 1992) have been reported to be suitable for production of secondary metabolites in different microorganisms. It was also reported that glucose, usually an excellent carbon source for growth, interferes with the biosynthesis of many antibiotics such as bacitracin (Haavik, 1974) and actinomycin (Gallo and Katz, 1972). During studies on fermentation medium development, polysaccharides or oligosaccharides are often found to be better than glucose as carbon sources for antibiotic production (Martin and Demain, 1980). Duration of fermentation and temperature also affected the biological activity of the metabolites extracted from EPB. Maximum antimicrobial activity was obtained when the bacterial fermentation was carried out for 24 h at 37 ºC. Earlier studies on *Bacillus* sp. associated with *Rhabditis* (*Oscheius*) sp. also showed that antibiotic activity was strongly influenced by growth medium, temperature and duration of fermentation time Siji *et al*.(2013).

Addition of $MgSO_4$, $MgCl_2$, NaCl, KH_2PO_4, KNO_3, $(NH_4)_2SO_4$ favoured the production of antibiotic activity in *Xenorhabdus* sp. D43 whereas $Zn(NO_3)_2$ and $CuSO_4$ had a negative impact Wang *et al*., (2011). This revealed that trace elements in the media also play an important

Table 1. Comparative influence of peptone with different carbon sources on antifungal activity of culture filtrate solvent extract of *Enterobacter* sp.

Fungi	Time (h)	Zone of inhibition in (mm) for each combination*				Control	
		Peptone - glycerol	Peptone- maltose	Peptone - fructose	Peptone - glucose	Carbend- azin	Amphote- ricin
A. flavus	24	8	8	7	5	25	-
	48	7	6	7	5	24	-
	72	7	6	6	5	25	-
	96	0	6	9	0	25	-
	120	5	0	0	0	24	-
C. albicans	24	10	10	11	5	-	23
	48	10	9	6	5	-	22
	72	9	9	6	5	-	23
	96	0	8	6	0	-	21
	120	7	0	0	0	-	23
F.oxysporum	24	14	14	0	10	16	-
	48	10	12	6	9	15	-
	72	15	9	6	9	16	-
	96	10	15	5	0	15	-
	120	10	0	0	9	16	-
P. expansum	24	20	11	6	10	24	-
	48	10	10	6	10	22	-
	72	11	8	9	8	23	-
	96	0	10	9	8	24	-
	120	7	0	6	0	24	-
R. solani	24	14	12	7	10	19	-
	48	11	11	10	7	18	-
	72	12	11	10	10	19	-
	96	0	10	10	0	18	-
	120	8	0	0	0	19	-

*Values represent mean of three replications, - not tested (pd”0.05)

Table 2. Comparative influence of peptone with different carbon sources on antibacterial activity of culture filtrate solvent extract of *Enterobacter* sp.

Bacteria	Time (h)	Zone of inhibition in mm for each combination*					
		Peptone - glycerol	Peptone- maltose	Peptone - fructose	Peptone - glucose	Peptone - sucrose	Ciproflo-xacin
B. subtilis	24	12	10	3	5	4	31
	48	11	8	7	5	0	31
	72	5	7	8	5	0	31
	96	5	5	5	9	0	31
	120	0	4	0	0	0	31
E. coli	24	7	12	11	0	6	28
	48	6	9	9	3	0	28
	72	5	6	8	5	0	28
	96	5	5	5	7	0	28
	120	0	0	4	0	0	28
P. aeruginosa	24	13	11	4	4	5	25
	48	12	7	5	4	0	25
	72	14	7	5	5	0	25
	96	6	5	5	6	0	25
	120	0	6	0	0	0	25
S. aureus	24	8	8	11	5	7	31
	48	7	9	10	5	0	31
	72	7	6	8	5	0	31
	96	5	5	4	7	0	31
	120	0	0	4	4	0	31

* Values represent mean of three replications, (pd”0.05)

role in antimicrobial activity. Concentration of free amino acid proline in the hemolymph of *G. mellonella* plays an important role in the virulence and antibiotic activity of the EPB (Waterfield, 2004). In this investigation also variation in antibiotic activity was observed when the media were supplemented with different concentrations of L-Proline. The study confirmed that media with optimal levels of carbon, nitrogen sources, trace elements and amino acids play an important role in antimicrobial activity of EPB associated with the *Rhabditis (Oscheius)* sp.

Table 3. Antifungal activity of culture filtrate solvent extract of *Enterobacter* sp. at different temperature

Fungi	Zone of inhibition (mm)*											
	25 °C		30 °C		35 °C		37 °C		40 °		Carbe-ndazin	Ampho-tericin
A. flavus	.	20	5	15	5	18	7	18	.	18	25	.
C. albicans	5	20	10	22	10	23	11	33	.	26	.	23
F. oxysporum	5	30	11	35	11	37	12	45	5	38	16	.
P. expansum	.	25	12	25	12	26	13	30	8	25	24	.
R. solani	.	25	11	25	11	26	13	30	9	26	19	.

* Values represent mean of three replications, - not tested (pd”0.05)

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