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Optimization of Media 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. Media for the production of the bioactive metabolites were standardized with tryptic soya broth (TSB) supplemented with six carbon sources viz., glycerol, maltose, fructose, glucose, sucrose and lactose. Antimicrobial activity was found highest for culture filtrate solvent extract (CFSE) obtained from TSB plus glycerol combination. Among the different carbon sources tested, glycerol proved to be the best for antimicrobial activity and that was followed by maltose, fructose and glucose. Media with lactose and sucrose combination had the least antimicrobial activity. A high degree of variation in the level of antimicrobial activity against the test organism viz., *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634, *Penicillium expansum* MTCC2006, *Aspergillus flavus* MTCC 183, *Candida albicans* MTCC 277 was observed. The present study revealed that the source of carbon has a pivotal role in the production of antimicrobial activity.

Key words: Antimicrobial activity, entomopathogenic, *Rhabditis* (*Oscheius*), tryptic soya broth

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). *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). Red ants have been found to be effectively killed by the EPN in the field. (Mohandas et al., 2007). These EPN were found to kill a number of important insect pests within 24-72 h in laboratory conditions.

The production of secondary metabolites with antibiotic properties is a common characteristic of EPB. Culture conditions are critical to the secondary metabolites production of microorganisms (Bode, 2009). Media such as yeast extract broth and its modifications, Luria Bertani broth (LB), sea water and tryptic soya broth (TSB) were used successfully for the production of antimicrobial metabolites from EPB. (Paul et al., 1981; Akhrust, 1982; Sundar and Chang1993; Li et al., 1997; Ji et al., 2004). The production of antimicrobial metabolites is strongly influenced by the culture medium and the fermentation conditions such as pH, temperature, agitation and oxygen availability (Fang et al., 2010). The antibiotic activity of *X. nematophila* was highly influenced by carbon and nitrogen sources of the medium (Yang et al., 2001, Yang et al., 2006, Wang et al., 2008). Previous studies on *X. nematophila* also showed the strong influence of growth media, inoculum age, inoculum volume, pH, temperature,

fermentation time on cell growth and secondary metabolite accumulation. (Li et al., 1997, Fang et al., 2010). A high degree of variation in the level of antimicrobial metabolite production of *Bacillus* sp. associated with *Rhabditis* (*Oscheius*) sp. was reported by changing carbon and nitrogen sources in the fermentation media (Kumar et al., 2012). Earlier studies on *Bacillus* sp. associated with *Rhabditis* (*Oscheius*) sp. also showed that antibiotic activity was strongly influenced by growth media, temperature and duration of fermentation time (Vijayakumari et al., 2013). The influence of culture conditions including incubation time, incubation temperature, initial pH and different carbon and nitrogen sources on growth and production of bioactive compounds was reported by Bundale et al., (2015). The present study was conducted to optimise different culture media for the maximization of antimicrobial activity of *Enterobacter* sp. strain KA1 associated with *Rhabditis* (*Oscheius*) sp. for the production of novel bioactive metabolites.

Materials and Methods

Entomopathogenic bacteria and nematodes

The entomopathogenic bacterium used in this study was isolated from EPN belonging to the genus *Rhabditis* (*Oscheius*) sp. isolated from soil collected from kannur district (Kerala) and maintained at CTCRI. The bacteria was isolated from infected cadavers of *Galleria mellonella* 48-hour post infection according to the method as described by (Woodring and Kaya, 1988) and the bacterial culture was maintained in nutrient agar by subculturing.

Fermentation media

Different standard media such as tryptic soya broth (TSB), Luria Broth (LB), nutrient Broth (NB) were used for fermentation. The TSB medium supplemented with different carbon sources such as glycerol, maltose, fructose, dextrose, sucrose, lactose each at a level of 1 % (w/v) was also used as media for fermentation.

Extraction 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 4º C and passed through a 0.45 μM filter to obtain cell free culture filtrate.

Separation 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 40º C, and the metabolite obtained was weighed, reconstituted in methanol and used for assay of antimicrobial activity.

Measurement of bacterial culture growth

Cell growth was measured by the optical density (OD) of the culture at 600 nm using double beam spectrophotometer. The biomass yield of crude extract of organic fraction (mgl^{-1}) and pH of the culture filtrate was also measured at different incubation period.

Antimicrobial assay

Antibacterial and antifungal activity of the CFSE studied were 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 Carbendazim 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

Influence of TSB, LB and NB media on culture growth

The effect of three different culture media such as TSB, LB, NB on bacterial growth was studied. The OD value was highest at 24 h for TSB and biomass yield was highest at 72 h (Table 1). The pH of the bacterial culture increased steadily from 7.0 to 8.2 upto 120 h and remained constant for the three media.

The bacteria showed maximum growth in TSB medium at 24 h (Fig. 1). As a result in variation in antimicrobial metabolite production by bacteria the pH increased from 7.2 on 24h to 8.22 upto 120 h (Fig. 2). The pH indicates the utilization of different carbon and nitrogen sources by the strain which directly influence the production of antimicrobial compounds. The growth and biomass yield of the bacterium in LB and NB media were significantly very low compared to TSB medium. (Table 1).

Influence of TSB with different sugars on culture growth

The effect of TSB with different sugars on bacterial growth was studied. The OD value was highest at 24 h for TSB plus glycerol and biomass yield was highest at 24 h. The growth of the bacteria in media supplemented with sucrose and lactose was low compared to carbon sources. The pH of the bacterial cell free culture filtrate decreased steadily from 7.0 to 6.87 upto 96 h and then increased to 7.69 upto 120 h for TSB plus glycerol medium (Fig. 3). The variation of pH value occurs due to difference in utilization of carbon sources by the bacteria.

Influence of TSB with different sugars on antimicrobial activity

Antimicrobial activity of the CFSE obtained from TSB with glycerol was significantly higher followed by TSB plus maltose, fructose, glucose, sucrose and lactose at 30 °C for 24 h (Table 2), and the highest antifungal activity

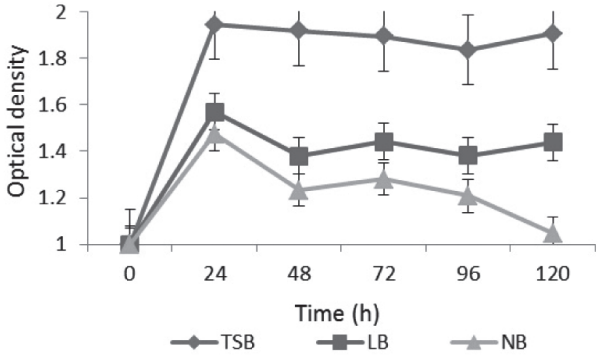

Fig.1. Bacterial growth on TSB, LB and NB at different time intervals

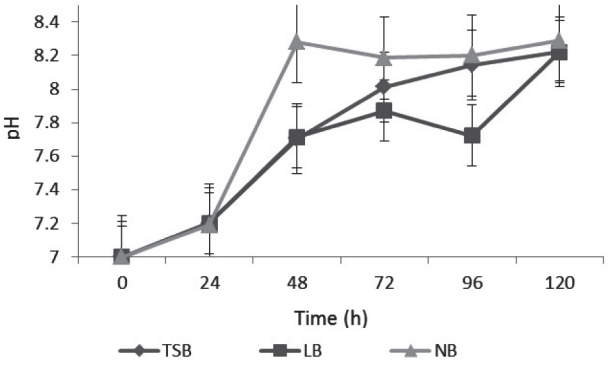

Fig.2. pH change of culture filtrate obtained from TSB, LB and NB at different time intervals

Table 1. Influence of TSB, LB and NB media on culture growth

Time (h)	TSB			LB			NB		
	OD value	pH	Yield (mg/L)	OD value	pH	Yield (mg/L)	OD value	pH	Yield (mg/L)
24	1.9^{b}	7.2^{a}	335^{d}	1.5^{c}	7.2^{a}	69^{a}	1.4^{c}	7.1^{a}	32^{a}
48	1.9^{b}	7.7^{b}	315^{c}	1.3^{a}	7.7^{b}	107^{b}	1.2^{b}	8.2^{b}	42^{a}
72	1.8^{b}	8.0^{d}	465^{e}	1.4^{b}	7.8^{b}	99^{b}	1.2^{b}	8.1^{b}	60^{c}
96	1.8^{a}	8.1^{d}	293^{b}	1.3^{a}	7.7^{b}	148^{d}	1.2^{b}	8.2^{b}	50^{b}
120	1.9^{b}	8.2^{c}	223^{a}	1.4^{b}	8.2^{c}	136^{c}	$1.^{0a}$	8.2^{b}	36^{a}

*Values represent mean of three replications ($p < 0.05$)

Table 2. Comparative influence of TSB with different carbon sources on antifungal activity of EPB

Fungi	Time (h)	Zone of inhibition in (mm) for each combination*						Control	
		TSB glycerol	TSB maltose	TSB fructose	TSB glucose	TSB - sucrose	TSB -lactose	Carben-dazin	Ampho tericin
A. flavus	24	14	14	8	11	7	5	25	
	48	13	12	13	9	12	5	24	-
	72	14	11	7	14	19	6	25	-
	96	10	10	5	7	13	4	25	-
	120	10	11	4	9	10	6	24	-
C. albicans	24	19	20	11	13	4	10	-	23
	48	16	13	10	8	3	6	-	22
	72	17	9	9	11	3	7	-	23
	96	10	10	5	6	5	4	-	21
	120	9	13	6	8	3	5	-	23
F. oxysporum	24	28	21	15	12	6	14	16	-
	48	18	21	11	11	0	5	15	-
	72	27	9	9	11	0	6	16	-
	96	9	9	6	8	0	6	15	-
	120	8	9	4	10	5	8	16	-
P. expansum	24	24	17	13	17	6	14	24	-
	48	17	21	9	14	4	5	22	-
	72	13	12	11	11	4	6	23	-
	96	9	12	7	6	4	5	24	-
	120	5	9	8	12	8	5	24	-
R. solani	24	17	16	18	13	6	11	19	-
	48	21	17	8	12	5	6	18	-
	72	23	13	9	11	4	5	19	-
	96	8	10	7	8	5	7	18	-
	120	6	9	6	12	0	3	19	-

*Values represent mean of three replications, - not tested ($p<0.05$)

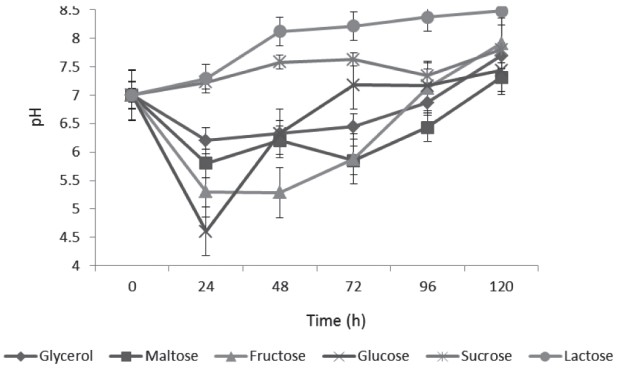

Fig.3. pH change of culture filtrate obtained from TSB plus different sugars at different time intervals

was observed against *Fusarium oxysporum*. The antibacterial activity was recorded highest in the CFSE obtained from TSB with glycerol and the antimicrobial activity of CFSE obtained from medium with lactose as carbon source was insignificant against the test organisms (Table 3).

As for the various carbon sources tested, maximum antimicrobial activity was obtained for TSB glycerol combination. The antimicrobial activity was very low for lactose and sucrose combination. Lactose is not utilized by the bacteria and sucrose also not favored for the production of antimicrobial metabolites. TSB plus glycerol medium was selected as the best medium for maximum production of antimicrobial metabolites. Variation in the fermentation conditions often results 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 the 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).

Table 3. Comparative influence of TSB with different carbon sources on antibacterial activity of EPB

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

*Values represent mean of three replications ($p<0.05$)

It was also reported that maltose and glycerol had the strongest effect on the antibiotic activity of *Xenorhabdus* sp. D43 (Yang et al*.,* 2006). 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 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 the 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. 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 (Vijayakumari et al., 2013). The study confirmed that media with optimal levels of carbon sources play an important role in antimicrobial activity of EPB associated with the *Rhabditis (Oscheius)* sp.

Conclusion

The present study reveals that changes in carbon, nitrogen sources and trace elements in the media will not only influence the growth and metabolism of the EPB but also increase the production of antimicrobial metabolites. So it is necessary to develop optimum conditions for the production of novel metabolites. Moreover the bacteria associated with the EPN posses enormous potential to produce novel antimicrobial metabolites. These

metabolites produced by EPB have a wide range of bioactivities with medicinal and agricultural interests such as antimycotic, antibacterial, insecticidal and nematicidal properties.

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