RESEARCH PAPER

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Enhancement of growth and biological activity of selected actinomycetes strains of *Melissa officinalis* and *Heracleum candicans* on different media

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Actinomycetes strains isolated from rhizosphere of two important medicinal plants *Melissa officinalis* and *Heracleum candicans* were evaluated for production of biological and proteolytic activities by selecting different media. In present investigations two strains Act-M-3 and Act-M-5 of *Melissa officinalis* produced maximum growth on Glucose ammonium salts (GAS) and Glycerol peptone beef (GPB) broths while strain Act-M-8 preferred GPB. Antibacterial and antifungal activities were registered more on GAS, GPB in addition to Nutrient broth (NB) against *Bacillus subtilis*, *Alternaria* and *Pythium* sp. by two strains Act-M-3 and Act-M-8 in comparison to Act-M-5. Proteolytic production was registered highest in Starch Broth (SB) by Act-M-3 than other two strains. However, Act-H-2 strain isolated from *H. candicans* recorded maximum growth on GPB while Act-H-5 and Act-H-6 obtained higher production on SB. GAS broth supported greater antibacterial activity by Act-H-2 and Act-H-6 strain towards *E. coli* and *B. subtilis* but less or weak effect was obtained by Act-H-5 strain in all the media tested. Antifungal effect against *Pythium* and *Phytopthora* sp. was found superior in GPB, GP, GAS and SB. Though proteolytic activity produced by Act-H-2 and Act-H-6 was more on SB, NB and GAS media. Strain Act-H-5 on other hand could not show any proteolytic production on GAS, GPB and SB.

Key words : Microflora, Nutritional selection, Secondary metabolites

INTRODUCTION

Actinomycetes constitute a significant component of microbial population in most soils and count over one million in gram of soil. Soil is also the most prolific source for their multiplication. According to Williams *et al.* (1983) over 200 genera have been isolated with Streptomycetes being ubiquitous and most numerous. Several reviews of soil Streptomycetes are available (Kuster and Williams, 1964; Kutzner, 1981; Lacey, 1973). Isolation of actinomycetes from mixed microflora is a complicated process due to their characteristics slow growth relative to other bacteria. It is resulted in the development of selective isolation procedure based primarily on nutritional selection in which media are formulated with nutritional selection which is preferentially utilized by actinomycetes (Kuster and Williams, 1964 ; Kutzner, 1981). This also depends on selective inhibition where compounds such as antibiotics are incorporated into media to selectively inhibit non-actinomycetes bacteria (Dulaney *et al.*, 1955; William and Devis, 1965; Preobrazhenshaya *et al.*, 1978; Kutzner, 1981). Nutrients and growth normally influences secondary metabolite production by microbes. Production of secondary metabolites was normally favored by providing the carbon and nitrogen sources in the complex form such as corn steep liquor, so that their release to metabolism did not encourage a rival increase in biomass. All secondary metabolites can be expected to exhibit biological activity because they are formed from primary intermediates by the action of enzymes that confirm to biological principles. Free living microorganisms produce antibiotic that inhibit pathogens (Lynch, 1987). Betty and Duriraj, 1986 reported abundance of Actinomycetes strains particularly of Streptomyces in rice fields that suppress the pathogens (*Pyricularia oryzae* and *Xanthomonas compestris* pv. *oryzae*) of rice.

Actinomycetes protease are potentially of great economic importance in diverse fields, food, pharmaceuticals, tanning and detergents industries (Outtrup and Boyce, 1991; Gracia-Carreno, 1991). Proteases are enzymes which catalyze the hydrolysis of peptides bonds forming the primary structure of protein. Today, proteases are probably the most important class of industrial enzymes, with worldwide scales of 236 million US$ in 1981, rising to 242 million US$ in 1986, accounting for nearly 60 per cent of total enzyme sales, with 2/3rd of proteases produced commercially by microorganisms (Kalisz, 1988).

The present study was thus carried out to determine the effect of different media on biological action of

Actinomycetes strains isolated from rhizosphere roots associated with *Melissa officinalis* and *Heracleum candicans*.

MATERIALS AND METHODS

Effect of media on growth and production of biological activities :

From preliminary *in vitro* studies of screening against bacteria and fungi for actimicrobial and proteolytic activity. Six potentially active isolates out of twenty of Actinomycetes 3 each from *Melissa officinalis* (Act-M-3, Act-M-5 and Act-M-8) and *Heracleum candicans* (Act-H-2, Act-H-5 and Act-H-6) were chosen for growth and biological effect on media manipulations (Table 1 and 2). Nutrient agar, Potato dextrose and Skim milk agar plates were used for estimation of these activities initially in selecting the useful potential strains of Actinomycetes

The effect of different media was studied for all the six isolates of Actinomycetes. Five different medium nutrient broth (NB), Glucose peptone broth (GPB), Starch broth (SB), Glycerol peptone beef broth (GPBB) and glucose ammonium salt broth (GASB) were prepared under mild shake conditions at 28^{0}C for 7days. In 50ml of each medium 1ml of 7 days old cultures of Actinomycetes strains were inoculated in 250 ml Erlenmeyer flasks and incubated at 28^{0}C for 7 days under similar mild shaking conditions. Cell free cultures of supernatants of each Actinomycetes were procured by centrifugation at 10,000 rpm at 4^{0}C for 30 min. Antibacterial, antifungal and proteolytic activities were checked by well/spot plate assay method. For antimicrobial effect, cell free supernatant of actinomycetes were spotted on pre-poured test medium having a lawn of indicator bacteria made by subculturing 12 h old cultures bacteria of 0.5 OD at 540nm. The antifungal activity was judged by placing 100µl of cell free supernatant of each Actinomycetes in well cultured on media, having a lawn of indicator test fungi by adding 1ml of 4 days old culture into 25ml of cooled molten medium of each kind. Similar steps were followed for proteolytic assessment on different media. Inoculated plates were incubated at 37^{0}C for 24 hrs and 28^{0}C for 72hrs for antibacterial, proteolytic and antifungal expressions, respectively. All the three bioactivities were expressed in terms of mm of dia. of clear zone formation around the well/spot on their respective media plates. The experiment was subjected to analysis of variance techniques using Completely Randomized Design (Gomez and Gomez, 1976) with three replications in each case.

Table 1: Effect of media on growth of Actinomycetes strains isolated from rhizosphere of *Melissa officinalis*

Media (Broth)	Growth of *Actinomycetes* (After one week)		
	Act-M-3	Act-M-5	Act-M-8
Nutrient	0.64	0.84	0.69
Glucose peptone	0.63	0.42	0.60
Glucose smmonium salt	0.95	0.90	0.40
Glycerol peptone beef	0.51	0.90	0.73
Starch	0.44	0.25	0.48

The χ^2 stastistic for the data above is 9.38 with 4 degree of freedom

Table 2 : Antibacterial and antifungal activity of Actinomycetes strains isolated from rhizosphere of *Melissa officinalis* on different media

Media (Broth)	Antibacterial activity* (mm dia) (*Bacillus subtilis*)		
	Act-M-1	Act-M-6	Act-M-8
Nutrient	25.0	0.0	20.0
Glucose peptone	28.0	14.0	40.0
Glucose ammonium salt	25.0	12.0	40.7
Glycerol peptone beef	28.0	12.0	42.0
Strach	25.0	0.0	23.3

The χ^2 stastistic for the data above is 334.99 with 4 degree of freedom

Media (Broth)	Antifungal activity * (mm dia)		
	Alternaria sp.	*Phytopthora sp.*	*Pythium sp.*
	Act-M-3	Act-M-5	Act-M-8
Nutrient	27.3	0.0	18.0
Glucose peptone	22.7	17.0	21.3
Glucose ammonium salt	30.7	15.0	27.0
Glycerol peptone beef	22.7	15.0	16.7
Strach	25.3	0.0	17.3

The χ^2 stastistic for the data above is 281.02 with 4 degree of freedom

RESULTS AND DISCUSSION

The results obtained from the present investigation have been presented in the following sub heads:

Assessment of biological activities on different media of Actinomycetes strains isolated from of Melissa officinalis :

The growth of Act-M-3 and Act-M-5 maximized at 7 days of incubation in glucose ammonium salt broth (GA 5B) followed by Glycerol peptone beef in Act-M-5. Strain Act-M-8 also grew well in Glucose peptone beef broth.

Though in all the media starch did not supported the growth compare to other media compositions. Generally three media, GAS, Nutrient and Glycerol peptone beef were preferred by all the Actinomycetes isolates.

An antibacterial and antifungal activity was highest in GPB, GP, GASB and nutrient broth against *Bacillus subtilis*, *Alternaria* sp. and *pythium* sp. by Act- M-3 and Act-M-8 strains. While with strain Act-M-5 no activity was observed on nutrient and starch broth. Although GP also proved significantly superior to other tested media against *B. subtilis* and *Phytophthora* sp. as these did not supported much biological activity. The production of antifungal activity to *Alternaria* sp. by strain Act-M-3 was adjudged highest in GAS followed by nutrient broth. *Phytophthora* sp. was inhibited maximum in GP by Act-M-5 while *Pythium* was inhibited highest in GAS followed by GP in case of Act-M-8 strain. No difference in antibacterial and antifungal activity was observed by Act-M-5 towards *B. subtilis* and *Phytopthora* sp. grown on GAS and GPB medium. However, weak activity was registered in nutrient and starch medium.

The average proteolytic activity produced in case of Act-M-3 was in order of SB, GAS, GP and GPB and Nutrient both. The rest two strains Act-M-5 and Act-M-8 produced more activity in GP (Table 3).

Table 3 : Production of proteolytic activity of Actinomycetes strain. (*Melissa officinalis*)

Media (Broth)	Proteolytic activity* (mm dia)		
	Act-M-3	Act-M-5	Act-M-6
Nutrient	30.0	13.0	21.0
Glucose peptone	31.0	23.1	27.0
Glucose ammonium salt	32.0	21.0	22.7
Glycerol peptone beef	30.7	21.0	18.7
Starch	33.3	13.0	22.0

The χ^2 stastistic for the data above is 359.43 with 4 degree of freedom.

Table 4 : Growth of Actinomycetes strains isolated from rhizosphere of *Heracleum candicans* on different media

Media (Broth)	Growth (A_{540})		
	Act-H-2	Act-H-5	Act-H-6
Nutrient	0.70	0.23	0.33
Glucose peptone	0.78	0.17	0.31
Glucose ammonium salt	0.72	0.13	0.31
Glycerol peptone beef	0.81	0.31	0.33
Starch	0.69	0.34	0.41

The χ^2 stastistic for the data above is 6.57 with 4 degree of freedom

Biological activity in Actinomycetes isolated from Heracleum candicans :

The growth of strain Act-H-2, from *H. candicans* was recorded highest in GPB followed by starch broth in case of Act-H-5 and Act-H-6. The antibacterial activity against *E. coli* produced by Actinomycetes Act-H-2 at 7 days of incubation was maximum in three media GP, GAS and starch broth as compared to GPB and nutrient broth (Table 4). However, Act-H-6 showed significantly more production of antibacterial effect toward *Bacillus subtilis* in GAS followed by nutrient broth. The strain, Act-H-5 on contrast indicated less or weak effect towards antibacterial activity, but exerted some antifungal effect against *Phytophthora* and *Pythium* spp. on GP and GAS medium. Whereas GPB was significantly more active in suppressing the *Pythium* pathogen by Act-H-2 strain than rest of the media. However, GAS in addition to SB was found better in increasing antifungal effect associated with strain Act-H-6 as both produced statistically same effect (Table 5).

The production of proteolytic activity by Actinomycetes such as Act-H-2, Act-H-5 and Act-H-6 revealed that strains Act-H-2 and Act-H-6 preferred Starch broth. Maximum (32.0 and 30.67 mm), although GAS also was found statistically at par in case of Act-H-6 strain while strain Act-H-5 grew best in nutrient broth

Table 5 : Antibacterial and antifungal activity of Actinomycetes strains isolated from rhizosphere of *Heracleum candicans* on different media

Media (Broth)	Antibacterial activity* (mm dia)			Antifungal activity*(mm dia)			
	E. coli		*B. subtilis*	Act-H-2	Act-H-5		Act-H-6
	Act-H-2	Act-H-5	Act-H-6	*Pythium* sp.	*Pythium* sp.	*Phytopthora* sp.	*Phytopthora* sp.
Nutrient	28.7	0.0	27.3	23.3	11.6	11.0	24.7
Glucose peptone	30.7	0.0	24.7	24.7	19.3	0.0	22.7
Glucose ammonium salt	30.7	0.0	30.7	27.3	14.6	15.0	25.3
Glycerol peptone beef	29.3	0.0	24.0	31.3	13.0	0.0	24.0
Starch	30.7	0.0	22.7	20.0	13.3	0.0	25.3

The χ^2 stastistic for the data above is 279.35 with 4 degree of freedom

followed by glucose peptone with no registration of this activity in glucose ammonium salt, glycerol peptone beef and starch broth media (Table 6).

Table 6 : Production of proteolytic activity of Actinomycetes strains of *Heracleum candicans* on different media

Media (Broth)	Proteolytic activity* (mm dia)		
	Act-H-2	Act-H-5	Act-H-6
Nutrient	31.3	25.0	27.3
Glucose peptone	31.3	21.0	28.7
Glucose ammonium salt	28.7	0.0	30.7
Glycerol peptone beef	25.3	0.0	24.7
Starch	32.0	0.0	30.7

The χ^2 stastistic for the data above is 336.67 with 4 degree of freedom

In all the strains, the average activity was recorded upto 7 day of incubation and was found significantly highest compared to alternative days of date recording.

Manipulation of media and growth conditions of microorganisms is a common strategy used by pharmaceutical companies to improve the therapeutic interest. Secondly metabolites production in microbes is strongly influenced by nutritional factor and growth condition (Tormo *et al*., 2003). The manipulation of substrate like C, N sources restrict the growth rate by controlling the ion or oxygen uptake may also lead to significant improvement (Flickinger and Perlman, 1979 and Vandamme, 1984). Cultural conditions play an important role in culture growth and in production of biological activities by microorganisms (Singh *et al*., 1983) as the physiological and nutritional requirement of an organism is genetically predetermined. Although good growth may occur in many media but secondary metabolites may only be produced in a specific medium (Bentley *et al*., 1962). Sometime a good organism may produce one metabolite in one medium and totally different in other medium (Oxford *et al*., 1935). The presence or absence of certain ion or C, N sources can inhibit, activate, induce and depresses certain enzymes, perturbing the normal channeling of key intermediates that supports the balance growth (Malik, 1990). Humic acid vitamin (HV) agar used as selective medium for isolation of endophytic actinomycetes of plant spp. *Streptomyces* spp. were identified by Taechowisan *et al*. (2003), which inhibited *Colletotrichum mosae* and found very effective against *F. oxysporum*. Iznaga *et al*. (2004) obtained different 563 strains of actinomycetes from Cuban soils out of which 286 produced compounds with antifungal activity. The disk assay screening method indicated the presence of many possible polyene macrolide antibiotics and an increase in antifungal activity in soil rich in mineral. Khamna *et al*. (2008, 2009) obtained 89% of actinomycetes isolates belong to genus *Streptomyces* and 11% to non-*Streptomyces* sp. isolated from medicinal plant rhizosphere soils. Of the total, 23 isolates showed antifungal effect towards 5 phytopathogenic fungi *Alternaria brassicicola*, *A. porri*, *Collectotrichum gloeosporiodes*, *Fusarium oxysporum*, *Penicillium digitatum* and *Sclerotium rolfsii*. However, 36 isolates were able to produce indole-3-acetic acid and 75 produced siderophore on chrome-azurol S (CAS) agar. Two strains of *Streptomyces* CMU-PA101 and Streptomyces CMU-SK126 had high ability to produce antifungal compound, IAA and siderophores. Prapagdee *et al.* (2008) obtained strains of Actinomycetes SRA14 of *Streptomyces hygroscopicus* from rhizospheric soil that produced extracelluar chitinase and β 1-3 glucanase during the exponential and late exponential phases, respectively. These were found effective on antagonizing *Colletotricum gloeosporioides* and *Sclerotium rolfsii*, the possible growth suppression due to enhanced extracellular metabolite. Percentage of growth inhibition by the stationary culture filtrate was significantly higher than that of exponential culture filtrate.

Similar effects were observed in the present study which was focused in the identification of a suitable growth medium for selective potential yielding strains of *Actinomyces* spp. for enhancing antimicrobial and proteolytic activity. It has been observed that six strains, three Act-M-3, Act-M-5 and Act-M-8 isolated from *M. officinalis* and three strains Act-H-2, Act-H-5 and Act-H-6 from *H. candicans* could induce higher level of antibacterial, antifungal and proteolytic activities in addition to maximum growth during the fermentation if grown on appropriate medium. Several strains of actinomycetes from rhizospheric soil antagonized *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *Streptomyces* sp., *Saccharomyces cerevisiae*, *Candida utilis* and *Aspergillus niger* (Parvateesam and Bulchandani, 2003; Marilen *et al*., 2007). The results of present study showed that the isolates (Act-M-3, Act-M-5, Act-M-8) preferred different media for the optimum growth and production of antimicrobial activity. Higher growth was obtained in GPB and GAS media while production of antibacterial activity of *M. officinalis* toward *Bacillus subtilis* was recorded in glycerol peptone beef and glucose peptone by Act-M-8 and Act-M-3 than Act-M-5 which also preferred GAS. The two media GAS and GP found supportive to *Alternaria* and *Pythium* spp. by giving more antifungal effect. This may be due to large extent on the metabolic characteristics of culture. Similarly from *H. candicans*

isolates Act-H-2, Act-H-5 and Act-H-6 registered more growth on GPB and strach broth was same. Antibacterial effect in Glucose ammonium salt, Glycerol peptone and SB against *E. coli* and *B. subtilis* was strong in Act-H-2 and Act-H-6 but a weak effect was reported with Act-H-5. However antifungal activity was more in Act-H-2, Act-H-6 strains in comparison to Act-H-5 on GPB, GAS and SB. The presence of glucose, glycerol and ammonium ions was reported to induce higher production of metabolities against *Pythium* and *Phytopthora* sp. as advocated by Martin *et al.* (1977*)*. Strain Act-H-2 was effective to *Pythium* while Act-M-6 showed more antagonism to *Phytopthora* sp. Difference in production behavior of metabolites may be attributed by metabolic and genetic characteristics of the strains. As per Hopwood (1988) antibiotics are metabolites that are biosynthesized by coordinated action of several genes for ensuring protection from the inhibitory effects of the producing organisms itself. Often culture produce more than one antibiotics simultaneously each needing its own structural, regulatory and resistance genes (Vandamme, 1984; Berwick, 1988). Antibiotic productivity depends on the maintenance of sub optional growth through control of the concentration of inorganic phosphate, ammonium ions, metals ion, carbon, nitrogen and oxygen (Bu'Lock, 1961).

Glucose is generally used in the fermentation as a preferred carbon source for antibiotic production but catabolic repression is avoided by feeding glucose during the fermentation. Lactose and starch also found better carbon source for antibiotic production (Martin *et al.*, 1977). Ammonium ions on other side exert a negative effect on production of several antibiotics such as cephamycin clavulenic acid, streptomycin and in commercial fermentations use of a slowly metabolized nitrogen source partially overcome this problem. The biochemistry of ammonium repression is due to repression of some of the key enzymes such as valine dehydrogenase that has been well studied by several workers (Omura *et al*., 1983; Brana and Demain, 1988). The biochemical diversity of microorganisms make them logical source of a wide variety of enzymes for use in food and other biochemical systems. Physiological, metabolic and genetics of microorganisms increase the potential for production of enzymes (Taylor and Oremland, 1979). In the present study, the production of protease activity is different in all the three media. Strain Act-M-3 from *M. offcinalis* produced excellent amount of proteolytic activities in the all test media with maximum in starch broth, where as the strains Act-M-5 and Act-M-8 preferred glucose peptone broth, Starch broth, GAS. However, Nutrient broth and GP supported more proteolytic activity by Act-H-2, Act-H-6. While Act-H-5 strain but did not produced any protease activity in glucose ammonium salt, glucose peptone and starch broth which may be ascertained due to the metabolic and genetic characters of the strains. Chitinolytic and proteolytic activity of *Streptomyces* was observed in strains identified from root free soil rhizosphere and mycorrhizosphere soils. They were able to hydrolyse gelatin and sodium caseinate in agar media. Enrichment of these media with glucose and ammonium nitrate caused induction and stimulation of *Streptomyces* spp.

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