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OPTIMIZATION OF ANTIFUNGAL COMPOUND PRODUCTION BY A MODERATELY HALOPHILIC *STREPTOMYCES WERRAENSIS* HB-11

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ABSTRACT: A moderately halophilic strain of actinomycete HB-11, having ability to produce antifungal compound was isolated from soil samples collected from the region of Mumbai sea port, India. The strain HB-11 was identified as *Streptomyces werraensis* on the basis of its morphological characteristics and 16S rRNA gene sequencing. It is found to be active against both filamentous as well as yeast fungi including *Aspergillus parasiticus, Aspergillus niger, Aspergillus flavus, Ustilago maydis, Helminthosporium gramineum, Alternaria solani, Mucor liomelis, Rhizopus oryzae, Sclerotium rolfsii, Fusarium moniliforme, Fusarium oxysporum* and *Candida albicans*. The cultural conditions for the production of antifungal metabolite were optimized by using various parameters and using *U. maydis* as indicator test strain. The antifungal metabolite was extracted with organic solvents, purified by column chromatography and its polyene nature was determined by Ergosterol plate assay. The optimization studies for antifungal compound production revealed that the suitable period of incubation as 8 days, pH as 8.0, salinity (5%), starch and ammonium sulphate as suitable carbon and nitrogen sources respectively and presence of magnesium sulphate at 25mM in soybean casein digest broth.

INTRODUCTION: Emerging infectious diseases caused by fungi are recently recognized as a worldwide threat to food security and animal and human health. The pathogenic fungi challenge food security by decimating our harvests, causing widespread malnutrition and starvation [1, 2], they threaten extinction of wildlife, such as species of amphibians [3] and bats [4] whilst human pathogenic fungi cause debilitating disease or acute or fatal infections, particularly in immunocompromised patients [5]. The present strategies to control fungal pathogens include use of chemical fungicides in agriculture and synthetic antifungal agents for clinical pathogens.

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However, excessive use of chemical fungicides in agriculture is associated with deteriorating human health, environmental pollution, lack of specificity, slow biodegradation rates and accumulation, and development of pathogen resistance [6]. A high toxicity exhibited by fungal pathogens and their increasing drug resistance pattern are major factors that also limits the number of antifungal substances approved for human use in the drug market.

Further, many fungi are flexible in their ability to undergo genetic recombination, hybridization or horizontal gene transfer [7], causing the clonal emergence of pathogenic lineages and allowing the formation of novel genetic diversity leading to the genesis of new pathogens [8]. Additionally, the larger number of opportunistic infections in immunosuppressed patients, together with the growing resistance exhibited by these fungus species, poses a considerable challenge to the pharmaceutical industry in the search for safe and efficient antifungal drugs [9].

Secondary metabolites of microbial origin represent a major source of compounds with diverse chemical structures and remarkable biological activity [10]. Actinomycetes have a long track record of producing novel molecules of biological importance and over 10000 of such compounds are reported from different genera of actinomycetes. However, the immense biotechnological potential of actinomycetes had led to extensive search on isolates from normal terrestrial habitats and found an associated increase in the number of compounds being rediscovered due to a high rate of redundancy in the strains isolated [11]. As the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, much attention has been focused on screening of actinomycetes from diverse environments [12].

In this context, the actinomycetes of the marine ecosystems are viewed as a rich source of microorganisms capable of producing useful antimicrobial compounds [13] and compared to terrestrial species, marine actinomycetes are important sources of novel antibiotics [14]. Therefore, the marine actinomycetes are often screened for the production of novel metabolites and numerous metabolites have been isolated during the past decade. However, such screening protocols are still at their infantile and marine actinomycetes could still be exploited for production of novel antimicrobial compounds [15].

The ability of actinomycetes to form bioactive products is not a fixed property but can be influenced greatly under varying conditions of nutrition and cultivation. Complete knowledge of optimal conditions required for maximum fermentation activity leading to antimicrobial metabolite production by actinomycetes is required so as to standardize the different physical and physiological factors influencing the production of metabolites having antibiotic properties with particular reference to the strain used [16]. In view of this, the present research work deals with the isolation of actinomycetes strains from marine soil samples collected from Mumbai Sea port, India, identification of a potent strain capable of producing antifungal compound at species level and optimization of conditions required for production of antifungal compound.

MATERIALS AND METHODS:

Microorganism:

The moderately halophilic actinomycete strain HB-11 was isolated from soil collected from Mumbai Sea Port (18°56.3' N latitude and 72°45.9'E longitude), on the natural deep water harbor of Mumbai, India. The soil (10g) was air dried for 72h, suspended in sterile distilled water (100 ml) and serially diluted (10^{-2}, 10^{-3} and 10^{-4}) after shaking for 30 min in an orbital shaker incubator. The dilutions were spread on starch-casein agar[17] (SCA) supplemented with NaCl (10%), nystatin (50µg/ml) and nalidixic acid (30µg/ml). After an incubation of 10 days at 28°C, a typical grey colony of strain HB-11 was picked up, ensured for purity by re-streaking and maintained at 4°C as stock cultures on SCA slants.

Characterization of HB-11:

Morphology and sporulation pattern of HB-11 was observed under light microscope using inclined cover slip culture techniques [18] on SCA after incubating at 30°C for 7 days. Color of spore mass and aerial and substrate mycelia were recorded using National Bureau Standards (NBS) color name charts[19]. Chemotaxonomic studies were done by analyzing the whole cell hydrolysates for amino acids and sugar content [20, 21]. Further identification of the HB-11 isolate was carried out at molecular level by 16S rRNA sequencing. The DNA was isolated from HB-11 cells using the phenol chloroform extraction method [22] and used as template for PCR analysis using primers 8F: 5'AGAGTTTGATCCTGGCTCAG-3' and 907R: 5'-CCGTCAATTCMTTTRAGTTT-3'.

The amplification conditions were 94°C for 3 min, 52°C for 45 sec, 72°C for 1 min and final extension step of 72°C for 3 min. The PCR product was purified by PEG-NaCl method and sequenced by using ABI 3730XL sequencing machine. The forward and reverse sequences were assembled through DNA Baser V35.0 software and aligned with other closely related taxa retrieved from Gen Bank database. Comparison of 16S ribosomal RNA gene sequence of HB-11 and other known bacterial sequences was done using NCBI BLASTn and 772

bp sequence obtained was deposited in NCBI Gen Bank and its accession number was obtained. The reference sequences required for comparison were downloaded from http://www.ncbi.nlm.nih. gov/ genebank. The sequence alignment was done by using CLUSTAL W, a multiple sequence alignment program, gaps were removed manually and pairwise evolutionary distances were computed using the Kimura 2-parameter model [23]. The bootstrap analysis was done using 1000 replicates of original data set and used for constructing the phylogenetic tree in MEGA 5.2.2 using Neighbor joining method [24].

Antifungal activity of HB-11:

The actinomycete strain HB-11 was inoculated in 50 ml of starch casein broth and incubated for 7 days at 30℃ . After incubation, the culture broth was centrifuged at 15000 ×g for 20 min and cell free supernatant (CSF) was used to observe antifungal activity by agar well diffusion method [9]. The test organisms used were *Aspergillus niger* NCIM 836, *Aspergillus flavus* NCIM 542, *Aspergillus parasiticus* NCIM 904, *Alternaria solani* NCIM 888, *Ustilago maydis* NCIM 983, *Helminthosporium gramineum* NCIM 1070, *Mucor liomelis* NCIM 873, *Rhizopus oryzae* NCIM 878, *Sclerotium rolfsii* NCIM 1084, *Fusarium moniliforme* NCIM 1100, *Fusarium oxysporum* NCIM 1072 and *Candida albicans* NCIM 3471. The test fungi were spread over potato dextrose agar (HiMedia) plates.

Wells (5 mm) were made using sterile cork borer, and then 15 µl of the extract was added separately into each well and incubated at 28^{o}C for 24-48h. After incubation the diameter of the inhibition zone around the wells was measured. Each test was repeated three times and the antifungal activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite.

Production and purification of antifungal compound:

The HB-11 was grown in soybean casein digest broth (HiMedia) supplemented with 1% dextrose at 30^{o}C for 7 days and at 130 rpm on rotary shaker. At the end of incubation period, the fermented broth was centrifuged at 10,000 ×g for 30 min to separate biomass and used for the extraction of antifungal metabolite.

The effect of different solvents on extraction of antibiotic from supernatant was studied. Five different solvents such as acetone, ethyl acetate, chloroform, methanol and hexane were added individually to the cell free extract (1:1 v/v) and shaken vigorously for 15 min. The organic layers were collected and the solvents were evaporated to dryness in rotavapor to obtain crude antibiotic extract. All crude extracts were assayed for their antifungal activity against sensitive strain *U. maydis* using agar well diffusion assay. Respective solvents were used as control for comparison of activity. Methanol extraction of antibiotic was found suitable with respect to high yield and antifungal activity and hence this extract was used for further study.

The crude antibiotic was purified using silica gel (60-120mesh) column chromatography. The column was eluted with graded concentrations of methanol in water (0-100%) and fractions were collected in volume of 10 ml with a flow rate of 1 ml/min. Almost 30 fractions were collected and each fraction was tested for their antifungal activity against *U. maydis* using agar well diffusion assay.

The active fractions were analyzed for the number of components by thin layer chromatography using chloroform: methanol: water (65:30:5). After development, the spots of antibiotics were located with iodine vapors. The spots with similar Rf values were combined together after elution in methanol and further purified on silica gel column as described before.

Bioautographic analysis:

The antifungal activity of antibiotic was further confirmed by performing one dimensional bioautographic analysis. The TLC plate spotted with the column purified antifungal compound (1 mg/ 5 ml 65% methanol) was dried and sprayed with a spore suspension (≈10^{6} spores/ml) of test fungus *U. maydis*. The plates were incubated on a sheet of moist filter paper in a petri dish at 28°C for 48-72h. The diameter of the inhibition zone was noted and compared with the TLC for isolation of active constituents.

Ergosterol agar plate assay:
The Ergosterol agar plate assay described in the Laboratory Manual of MTCC [25] was used to detect the presence of polyene compounds in the antibiotic. *Candida albicans* was used as test organism. Sabouraud dextrose agar (SDA) plates with 0.5% ergosterol were prepared along with a control without ergosterol. The plates were seeded with the test organism. Wells were made with a sterile cork borer and 0.1ml of purified antibiotic (1mg/ml) was added to the well. The plates were incubated at 28°C for 24 h and observed for the zone of inhibition. Reduction in the size of inhibition zone over control plate was considered as an indication for presence of polyene compounds.

Optimization of process variables for growth and antifungal compound production:
Effect of incubation periods:
The SCDD broth was inoculated with HB-11 and incubated for 10 days at 30°C. At every 24h interval, the biomass was separated by centrifugation at 10,000 rpm for 20 min and dried at 70°C until a constant weight was obtained and expressed as mg/100 ml [26]. The methanol extract was prepared and tested for antifungal activity against sensitive strain, *U. maydis* by agar well diffusion method.

Effect of initial pH of medium:
The pH of the SCDD broth was adjusted to 4, 5, 6, 7, 8, 9 and 10 with 0.1M NaOH or HCl. All the flaks were inoculated with HB-11 and incubated at 30°C for seven days. The biomass and antifungal effect was determined as stated earlier.

Effect of carbon and nitrogen sources:
The basal medium soybean casein digest broth (SCCD) was supplemented with nine different carbon sources (10g/l) such as glucose, fructose, maltose, starch, xylose, sucrose, mannitol, cellulose and lactose and six nitrogen sources (5g/l) including yeast extract, malt extract, tryptophan, KNO_3, ammonium sulphate and peptone were inoculated separately with active culture of HB-11. The flasks were incubated for seven days under shaking conditions (130 rpm). After incubation, the extracts were tested for biomass and antifungal activities as mentioned before.

Effect of salinity and metal salts:
The SCDD broth was supplemented with NaCl (1-11%) and metal salts ($ZnSO_4$, $MgSO_4$, $MnSO_4$, $CaCO_3$, KH_2PO_4, K_2HPO_4 and $FeSO_4$; 25 mM). The flasks were inoculated with HB-11 and incubated at 30°C for seven days under shaking conditions. After incubation, the flasks were subjected for biomass and antifungal activity determination.

RESULTS AND DISCUSSION:
Characterization of HB-11:
The actinomycete strain HB-11 isolated from Mumbai port was identified to belong to *Streptomyces* genus on the basis of aerial and substrate mycelia formation and development of spiral spore chains. The isolate was gram positive and produced dark grey colonies on SCA with blackish colony reverse as represented in **Fig.1**.

The whole cell hydrolysate of HB-11 showed the presence of LL-Diaminopimelic acid and was without any characteristic sugar indicating a type I wall chemotype. Its further characterization was done on the basis of its 16S rRNA sequencing. The similarity level of query sequence of the isolate was best aligned (99%) with the existing species of *Streptomyces werraensis* NBRC 13404. The taxonomic position of the isolate was shown in **Fig.2.** The 16S rRNA sequence of the strain HB-11 as *Streptomyces werraensis* HB-11 has been deposited in Genbanks namely NCBI/EMBL under the accession number **KC710334**.

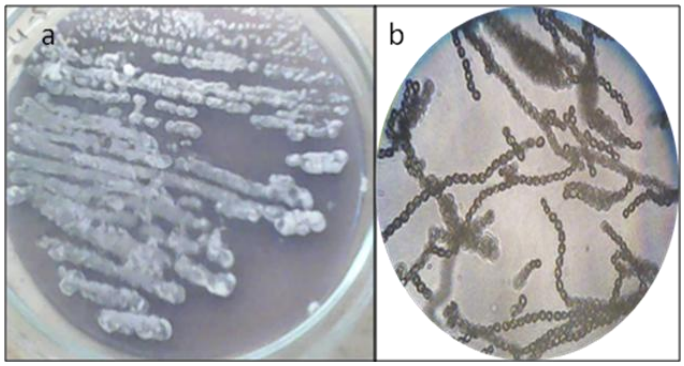


FIGURE 1A: LIGHT GREY COLONIES WITH DARK PURPLE DIFFUSIBLE PIGMENT OF *S. WERRAENSIS* HB-11 GROWN ON STARCH CASEIN AGAR
1B: LIGHT MICROGRAPH OF *S. WERRAENSIS* HB-11

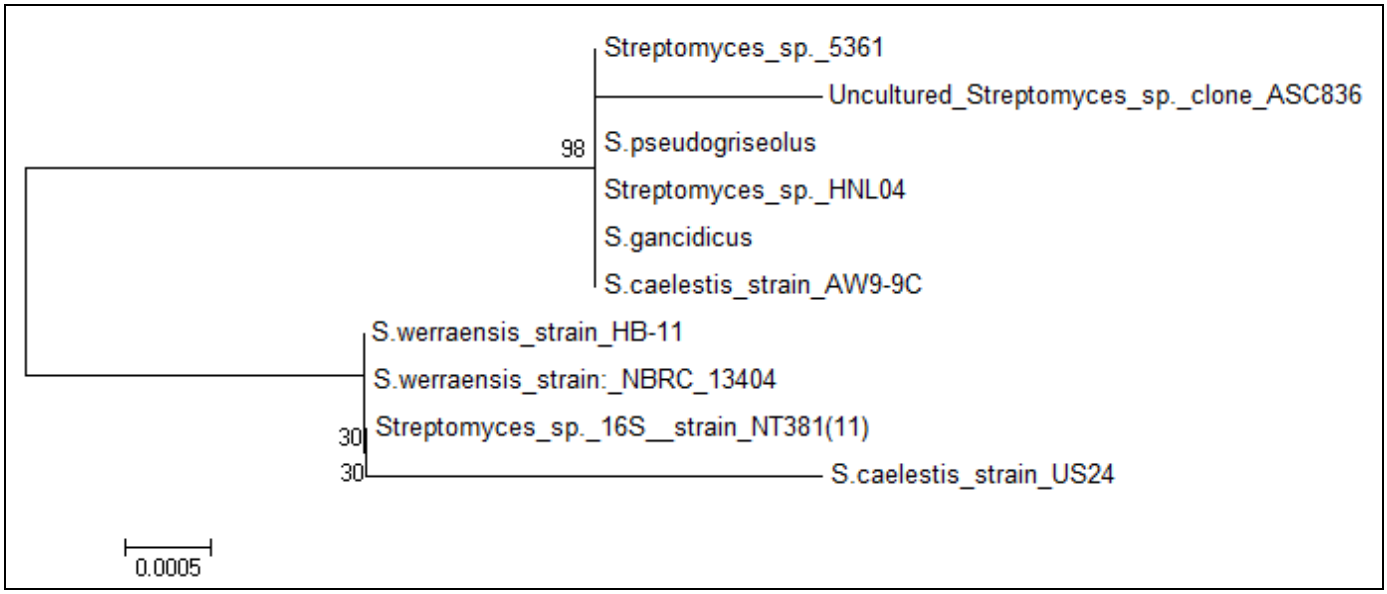


FIGURE 2: NEIGHBOR-JOINING TREE BASED ON 16S RRNA GENE SEQUENCE SHOWING RELATIONSHIP BETWEEN *S. WERRAENSIS* HB-11 AND RELATED MEMBERS OF THE GENUS STREPTOMYCES

Antifungal potential of *S. werraensis* HB-11:

The antifungal activity of *S. werraensis* HB-11 was screened against various fungal organisms. All test pathogens were sensitive to HB-11. The isolate showed highest zone of inhibition (30mm) against *Aspergillus parasiticus* NCIM 904 followed by *Aspergillus niger* NCIM 836 (27 mm) and *Ustilago maydis* (NCIM 983) (22mm). Least activity (10mm) was observed against *Sclerotium rolfsii* NCIM 1084 whereas other fungal test pathogens showed moderate sensitivity to *Streptomyces werraensis* **(Table 1 and Fig. 3)**.

TABLE 1: ANTIFUNGAL EFFICACY OF HB-11 CRUDE EXTRACT

Name of the test pathogen	Zone of inhibition (mm)
Aspergillus niger	27
Aspergillus flavus	14
Aspergillus parasiticus	30
Ustilago maydis	22
Helminthosporium gramineum	16
Candida albicans	15
Mucor liomelis	16
Rhizopus oryzae	13
Fusarium moniliforme	12
Fusarium oxysporum	13
Sclerotium rolfsii	10
Alternaria solani	12

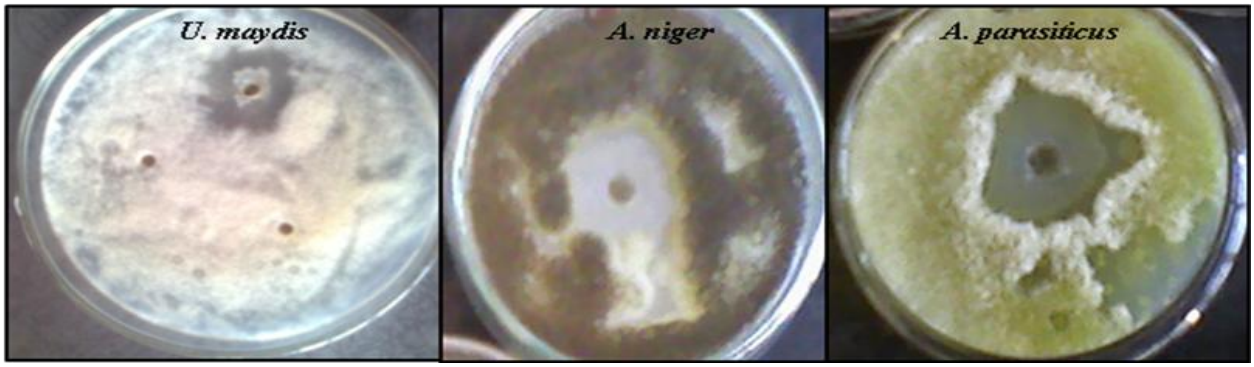


FIGURE 3: ANTIFUNGAL ACTIVITY OF *S. WERRAENSIS* HB-11 BY AGAR WELL DIFFUSION ASSAY.

Production and purification of antifungal compound:

In the present study, *S. werraensis* HB-11 could produce detectable quantities of antifungal compounds in soybean casein digest broth supplemented with 1% dextrose. The fermented broth of *S. werraensis* was extracted with five different solvents. The maximum antibiotic yield was obtained using methanol as extractant (0.84 mg/L) followed by ethyl acetate, chloroform and hexane **(Table 2)** whereas it could not be dissolved readily in acetone. The methanol extract was highly active against *U. maydis* and *A. niger* and produced

a maximum inhibitory zone of 23 and 21 mm respectively (data not shown). It has been reported earlier that the antimicrobial activity of the compounds from actinomycetes varied depending on the strains from which the compound obtained, the solvent used for the extraction and the nature of the pathogens tested against such compound [27, 28]. The extracted compound was purified and separated by column and thin layer chromatography. The fractions obtained from column were checked for their antifungal activity. The active fraction showed a single band on TLC plate and had Rf value of 0.32 cm.

TABLE 2: EXTRACTION OF ANTIFUNGAL ANTIBIOTIC FROM *STREPTOMYCES WERRAENSIS* HB-11.

Sr. No	Solvent	Zone of inhibition (mm)	Yield (mg/L)
1	Acetone	0	0.057
2	Methanol	28	0.840
3	Ethyl-Acetate	13	0.198
4	Chloroform	16	0.189
5	Hexane	21	0.194

Bioautography:

Bioautography technique used with spore forming fungi and bacteria is a very sensitive assay and gives accurate localization of active compounds [29]. In this technique TLC, plates are run and then the microorganism is introduced to the plate as a spray in the form of suspension in a growth medium and incubated. Inhibitory zones give indication of active compounds. The active fraction (Rf, 0.32 cm) isolated from *S. werraensis* showed inhibition of *U. maydis* (14mm) on TLC plate **(Figure 4)**.

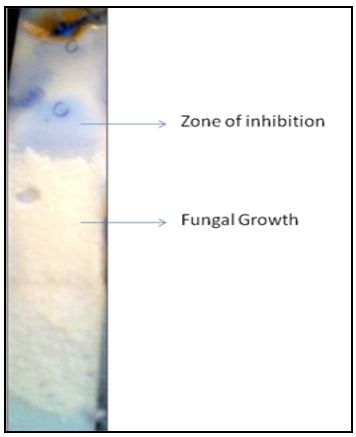


FIGURE 4: TLC BIOAUTOGRAPHY OF *S. WERRAENSIS* METHANOL EXTRACT WITH *U. MAYDIS* ON SILICA GEL G60 PLATE

Ergosterol plate assay:

Results of ergosterol plate assay showed the production of polyene antifungal antibiotic by *S. werraensis* HB-11. The antifungal compound obtained from its culture caused a zone of inhibition of 19 mm against *C. albicans* grown on Sabouraud dextrose agar medium without ergosterol whereas the same amount of sample when tested using SDA medium containing 0.5% ergosterol, produced only 12 mm zone of inhibition against *C. albicans*. Ergosterol and polyene compounds possess strong affinity and presence of ergosterol in assay medium resist the diffusion of polyene compounds [30]. This reduction in zone of inhibition in the presence of ergosterol indicated the production of polyene antifungal compound by the *S. werraensis* HB-11.

Optimization of process variables for growth and antifungal compound production:

The nutritional and cultural conditions are critical for the production of secondary metabolites by actinomycetes. Manipulating these conditions can promote biosynthesis of the secondary metabolites and facilitate discovery of new products. In the present study, various physical and chemical parameters had been optimized for the production of antifungal compound using *S. werraensis* HB-11. The effect of incubation period on growth and antifungal activity was recorded over a period of ten days.

The maximum activity (24mm) and growth (432mg/100ml) was observed after eight days of incubation. The activity of the isolate was observed from the second day of incubation and reached maximum after eight days (Figure 5A). Previous studies reported different incubation periods including, nine days [31], seven days [32], five days [33] and four days [34] optimum for growth and antimicrobial activity of *Streptomyces* sp. In fact, the ability of different *Streptomyces* sp to produce secondary metabolites in a specific incubation period depend on strain characteristics, available nutrients and presence of specific metabolic pathways.

Changes in the initial pH of the culture medium induce production of new substances that affect antibiotic production [35], hence, we examined the

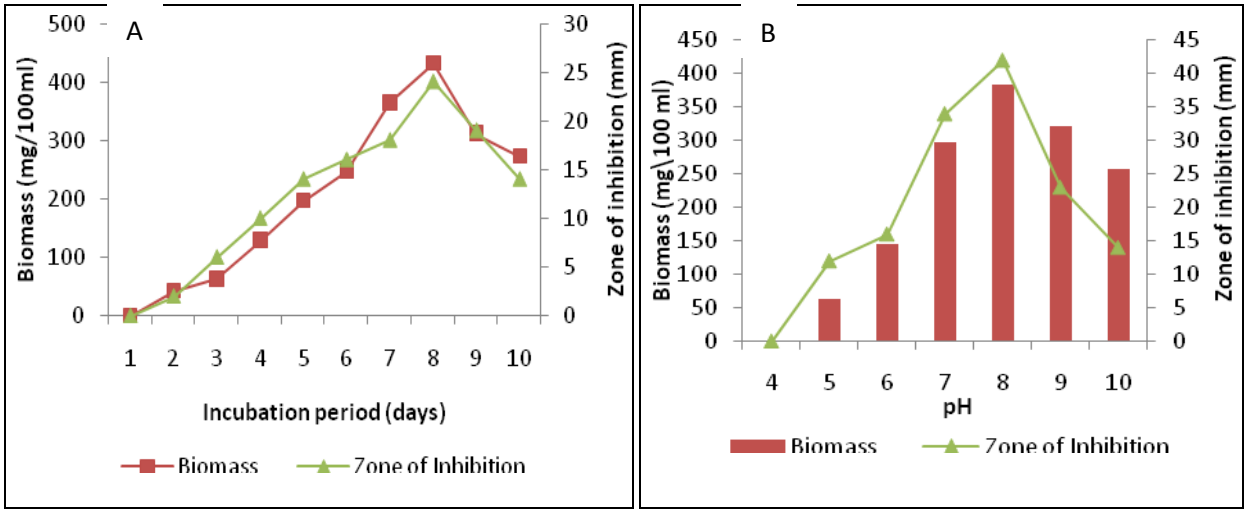
effect of pH on growth and antibiotic production by HB-11 and the results are presented in **Figure 5B**. The pH 8.0 was found to be the optimum for growth (384.3 mg/100ml) as well as antibiotic production (42 mm). This result is in accordance with most published literature where, optimum pH for antibiotic production in Streptomyces cultures has been reported to be near neutral [36, 15].

Antibiotic production by *S. werraensis* was measured by measuring the biomass and size of inhibition zones when the strain was growing on different carbon sources. The strain HB-11 showed maximum cell growth in medium supplemented with glucose but it was not found suitable for antibiotic production. Lactose was not used by the strain for appreciable amounts of biomass and antibiotic production. On the other hand, maximum antibiotic production was observed with complex carbon sources like starch **(Figure 5C).** Slowly assimilating complex carbon sources like polysaccharides are often known to stimulate secondary metabolite production as these substances do not cause catabolite repression in which production of enzymes of secondary metabolite biosynthesis is inhibited. Complex carbon sources are reported earlier to stimulate antibiotic production by actinomycetes as with starch [37] and cellobiose [38].

It is well known that changes in type and concentration of nitrogen sources affect antibiotic synthesis. In the present study also choice of nitrogen source affects antibiotic production by *S. werraensis* **(Figure 5D)**. Peptone was the best source for growth whereas the addition of ammonium sulphate in medium proved best for maximum antibiotic activity.

As shown in **Figure 5E**, the organism was able to grow and produce antibiotic at different NaCl concentrations. High NaCl concentration in the medium did not adversely affect the capability of organism to grow and produce antibiotic. The optimal growth was observed at a NaCl concentration of 8% whereas maximum antibiotic activity was observed at a concentration of 5%. Similarly, Vijaykumar et al.[15] reported maximum inhibitory effect of *Streptomyces* sp. VPTS3-1 at 4% salinity. In one more study, Kathiresan et al.[39], recorded higher antifungal activity of actinomycetes at 17.5 ppt of NaCl.

The addition of various metal supplements greatly affected the growth and antibiotic production by *S. werraensis* HB-11 **(Figure 5F)**. The maximum antibiotic activity (29 mm) was obtained when magnesium was supplemented at a concentration of 25mM followed by KH_2PO_4 (26 mm) and $ZnSO_4$ (24 mm). Similar trend was observed for growth of isolate. The salts of magnesium and potassium are considered the most appropriate salts for growth of Streptomyces as well as for production of active substance [40]. The stimulatory effect of magnesium is due to the requirement of magnesium for protein synthesis and restricting the enzyme synthesis in its absentia [41].



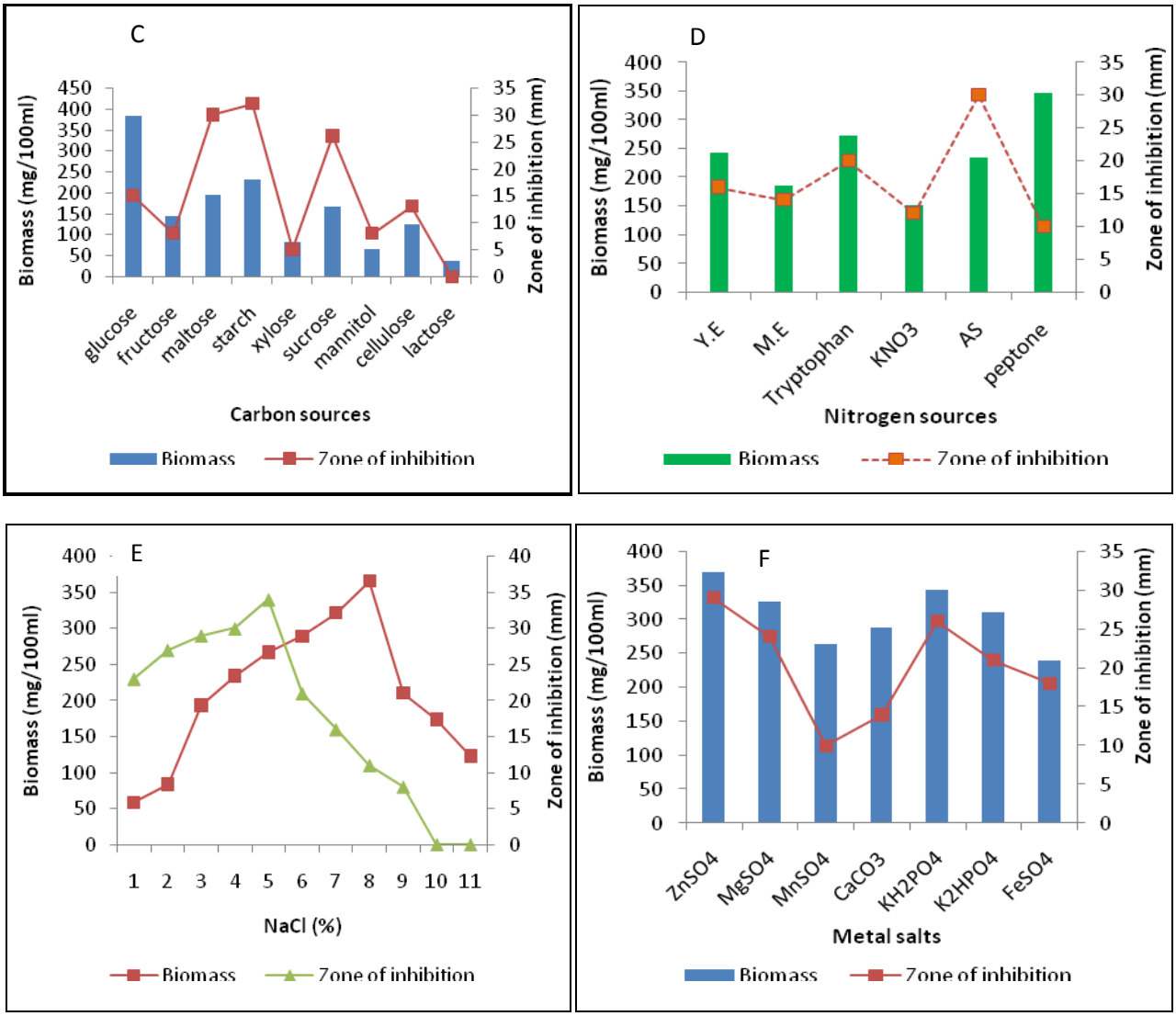


FIGURE 5: EFFECT OF INCUBATION PERIOD (A), INITIAL PH OF MEDIUM (B), CARBON (C) AND NITROGEN SOURCES (D), SALINITY (E) AND METAL SALTS (F) ON GROWTH AND ANTIBIOTIC PRODUCTION BY *S. WERRAENSIS* HB-11.

CONCLUSION: The results of present investigation revealed that a moderately halophilic strain of *Streptomyces werraensis* isolated from Mumbai sea port, India produces antifungal metabolites which are active against both filamentous and yeast fungi. This strain could be a potential source of novel antibiotics.

The study also showed that appropriate fermentation conditions including incubation time, pH, carbon and nitrogen sources, presence of proper metal salts and salinity of medium are required for optimal production of antifungal compound by *S. werraensis.*

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