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Isolation, screening and growth optimization of antagonistic *Bacillus subtilis* MS21 from Thengapattanam estuary against plant fungal pathogens

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Abstract

In the present work marine sediment samples were collected from Thengapattanam estuary on the south west coast of India for the isolation and screening of bacteria for biocidal potency. The density of the bacterial load in the sediment samples was ranged from $5.43x10^4$ to $2.8x10^6$ CFU/g. Screening of bacterial isolates for antagonistic activity revealed that the isolate MS21 had the highest zone of clearance among against all the 10 plant fungal pathogens tested. Among 10 fungal pathogens, the highest antagonistic activity was observed against *Gleosporium gleosporioide* (36mm) and the lowest against *Colletotrichum lamella* (11mm). The potential isolate was identified as *Bacillus subtilis* using biochemical methods and it was designated as *Bacillus subtilis* MS21. Effect of various physicochemical parameters on growth of *B. subtilis* MS21 showed that 36 hrs of incubation period, 150 rpm agitation, pH- 8.0, temperature-35^oC, salinity-1.0%, 2% sucrose as carbon source and 1% beef extract as nitrogen source were found to be the ideal conditions for maximum growth. Maximum growth of 1.92 OD was observed in mass scale culture with the optimized ideal conditions in the shake flask.

Keywords: Biocontrol, Biocide, Bioactive compound, *Bacillus subtilis*, Thengapattanam estuary, Marine sediment.

Introduction

Biological control is the reduction of pest populations by natural enemies also known as biological control agents that include predators, parasitoids and pathogens which suppress the pathogens by various mechanisms namely competition for nutrients notably iron, root colonization, secretion of lytic enzymes and antibiosis. Biological control, or the use of microorganisms or their secretions to prevent plant diseases, offers an attractive alternative for the control of plant diseases and also to reduce environmental pollution caused by chemical control. Therefore, biological control methods have become an important approach to facilitate sustainable agriculture (Martin and Hancock, 1987 and Wang *et al.*, 1999). Microorganisms used for biocontrol include bacteria, viruses, fungi and protozoa. Some of them are being used at commercia1 scales. Biocontrol agents have been used to control insect pests, weed and disease control.

Marine environment is the favourable habitat for of many groups of organisms and also is a complex environment of interactions among many organisms thriving there. The spectrum of direct interactions of

bacteria with marine organisms ranges from mutualism through commensalisms and competition, to antagonism, determined ultimately by balancing the cost of the association against the benefits received (Pinnock, 1994). Marine microorganisms are a new source of biologically active metabolites with novel chemical structures. The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals, and agrichemicals (Ireland *et al.*, 1993). Hence the present investigation was on the isolation, screening and growth optimization of a potent biocidal bacterium from sediment sample collected from Thengapattanam estuary on the south west coast of India.

Materials and Methods

Collection of sediment samples

Sediment samples were collected at 50cm-1m using a Petersen grab sampler in Thengapattanam estuary ($7^{o}53'$N latitude and $77^{o}07'$E longitude) on the south west coast of India. Samples were transferred to the laboratory immediately and kept at 4ºC until analysis.

Isolation and enumeration of Total Heterotrophic Bacteria (THB)

About 1g of sediment was aseptically weighed and transferred to a sterile conical flask containing 99 ml of sterile 50% aged seawater which was used as a diluent. The sediment- diluent mixture was agitated by means of mechanical shaking for about 5-10 min. and the samples were serially diluted up to 10^{-5} with sterilized 50% aged seawater and plated on Zobell marine agar (Zobell, 1941) medium (Hi-media, Mumbai) plates for the enumeration of total heterotrophic bacteria (THB) by adopting spread plate technique. Exactly 0.1ml of the serially diluted sample was spread over the sterile Zobell marine agar (ZMA) medium and the plates were incubated at 28±2 ºC for 24 to 48 hrs. The microbial load (density) in the given sample was calculated using the formula given below and it was expressed as Colony Forming Units (CFU) per gram of the sample. Total microbial load in the given sample ($CFU.g^{-1}$) = Total number of colonies/ Total volume of the sample x Volume of sample plated (0.1 ml) × dilution factor. Each morphologically different colony was isolated and streaked on ZMA slants and were maintained at $4^{o}C$.

Screening of bacterial isolates for antagonistic activity against plant fungal pathogens

Antagonistic assay was done by an agar-well diffusion method in aerobic condition. Isolated bacterial strains were tested for the antifungal activity. Phytopathogenic fungi such as *Macrophomina phaseolina***,** *Sclerotium roysii, Phytophthora infestans, Aspergillus niger, Alternaria alternata (Cotton), Gleosporium gleosporioide, Colletotrichum musae, Colletortichum capsici, Colletotrichum lamella* and *Rhizactonia solani* were spread on Potato dextrose agar plates. For spreading fungi, the fungal inoculums were prepared by inoculating a loopful of each fungus separately in a 5 ml of potato dextrose broth tube and incubated at 28ºC for 3 days till a moderate turbidity was developed. 100µl of the fungal culture was used for spreading. After spreading on agar surface 8mm wells were made with the sterile gel punch. For the inoculation and screening of antifungal activity of bacterial isolates; bacterial cell free culture broths was used. Bacterial cell free culture broths were prepared by inoculating each morphologically different colony in Zobell marine broth and incubated at 28±2 ºC for overnight. Cell free extract of each isolate was prepared by centrifuging the culture broths at 10000 rpm for 5 min. 50µl of cell free culture broth of each bacterial isolate used for screening. After an incubation period of 2-3 days at room temperature (28±2ºC), antagonistic activity was detected. The presence of zone of clearance around the wells on agar plates was used as an indicator for the antifungal activity. The strain which showed the maximum zone of clearance was chosen for further study. The presence of zone of clearance on agar plates was used as an indicator of bioactive potential of the strain (Portrait *et al.*, 1999).

Identification of bacteria

Isolates with different morphology were biochemically identified up to the species level by following Bergey's Manual of determinative bacteriology (Buchanan *et al.*, 1974).

Growth optimization of potential strain

Based on maximum antagonistic activity the isolate MS21 (identified as *Bacillus subtilis*) was selected for further growth optimization for the maximum

production of the biocontrol compound. Various physiochemical growth parameters, such as incubation period from 0 to 48hrs; different levels of agitation static condition, 50, 100, 150 and 200 rpm; different pH (i.e.) 5, 6, 7, 8, 9 and 10; various temperatures like 25°C, 30°C, 35°C and 40°C; different salinity range (varying concentration of NaCl) - 0.5%, 1%, 1.5%, 2%, 2.5% and 3%; different carbon sources such as maltose, sucrose, glucose, starch, and cellulose; different concentration of ideal carbon source (sucrose) from 1.0- 5%; different nitrogen sources such as peptone, beef extract, yeast extract, ammonium sulphate, ammonium nitrate and sodium nitrate and different concentration of beef extract as ideal nitrogen source from 0.5-2.5% were maintained in the medium. Growth was assessed for every 6 hrs up to 48hrs. Absorbance was measured at 600nm in a UV spectrophotometer (Systronics, Double beam spectrophotometer 2202) for every 6 hrs. The optimum parameter achieved by every step was fixed in the subsequent steps.

Mass cultivation in shake flask

The optimized growth conditions such as 36hrs of incubation, agitation -150rpm, pH-8.0, temperature-35°C, salinity-1.0%, sucrose-2.0%, beef extract-1.0% were maintained in the medium. Mass scale culture was done in 1L conical flasks with 0.75L of the medium. Growth was evaluated at the end of 36hrs incubation.

Results and Discussion

Biocontrol, using beneficial microorganisms to suppress plant diseases offers a powerful alternative to the use of chemical pesticides (Mizumoto *et al.,* 2007; Melnick *et al.,* 2008 and Ashnaei *et al.,* 2008). Biological control often uses microbial antagonists in combating plant diseases. To date most of the known microbial antagonists are isolated from the terrestrial environment. However, the number of novel microorganisms and products found in microbiologically poorly explored areas of the world suggests that a careful exploration of new habitats might continue to be useful. Marine environment, representing more than two thirds of our planet, is still under-explored and is considered to be an abundant resource for the isolation of less exploited microorganisms (Sponga *et al.,* 1999).
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Studies revealed that marine isolates produce antibiotic activities with frequencies exceeding the terrestrial ones (Burgess *et al.,* 1999). Microbial antagonists such as *Pseudomonas fluorescens, Agrobacterium radiobacter, Bacillus subtilis, B. cereus, B.amyloliquefaciens, Trichoderma virens, Burkholderia cepacia, Saccharomyces sp, Gliocadium* sp. have been successfully reported to possess antagonistic activities against plant fungal pathogens. However, there are only few publications are devoted to the study of the *Bacillus* species isolated from the marine environment. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats (Claus and Berkeley, 1986). A few Bacilli of marine origin have been reported to produce unusual metabolites different from those isolated from terrestrial bacteria (Jensen *et al.,* 1996). Yoon *et al.,* 2003; Yoon *et al.,* 2004 and Yoon and Oh (2005) isolated marine *B. aquaemaris* and *B. hwajinpoensis* respectively also *B. litoralis*. Miranda *et al.,* 2008 isolated *Bacillus* sp. from marine sediments.

Bacillus species have the ability to form endospores and synthesize a vast amount of metabolites, with the exception of toxin-producing *Bacillus anthracis* and *Bacillus cereus* and they are often considered beneficial and safe to plants and the ecological environment (Shoda, 2000). These properties of *Bacillus* species make them good biocontrol agents for substituting synthetic chemical fungicides. Among the *Bacillus* species, *Bacillus subtilis* is the most studied for its antagonistic activity and occasionally *Bacillus megaterium, B.cereus, B.pumilus and B.polymyxa* (Silo-Suh *et al.,* 1994). Shahzad *et al.,* 2017 studied plant growth-promoting endophytic *Bacillus amyloliquefaciens* RWL-1 which displayed antifungal activity against pathogenic *Fusarium oxysporum* f. sp. *lycopersici*.

In the present study, marine sediment samples collected from Thengapattanam estuary were spread plated on surface of the Zobell marine agar and the observed Total Heterotrophic Bacterial (THB) density in the sediment samples was ranged from $5.43x10^4$ to $2.8x10^6$ CFU/g (Fig. 1). Dhinakaran *et al.,* 2012 observed the bacterial density as $1.3x10^7$ CFU/g from the marine sediment samples of Mandapam in Gulf of Mannar area using Zobell marine agar. They also tested antifungal activity of a bacterial protein from marine *Corynebacterium sp.* of sediment origin.

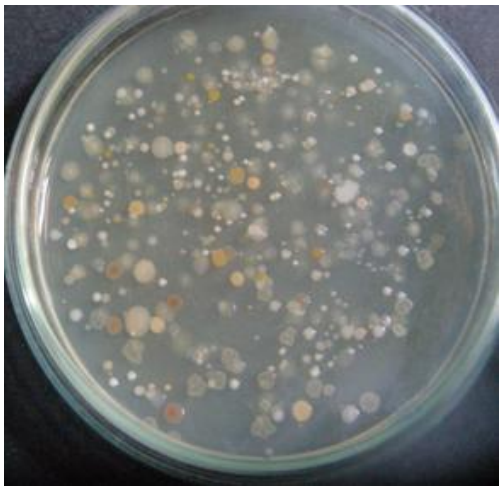

Fig. 1: Isolation of bacteria from marine sediment sample

Screening of bacterial isolates for antagonistic activity against plant fungal pathogens

In the present investigation the most potent strain was selected based on different morphology and the measurement of zone of clearance on using Well assay. Isolate MS21 (Fig. 2) was selected as the most potential as its cell free culture broth showed maximum zone of clearance to many of the plant fungal pathogens tested viz., *Macrophomina phaseolina* (27mm), *Sclerotium roysii* (16mm), *Phytophthora infestans* (28mm), *Aspergillus niger* (34mm), *Alternaria alternata* (28mm), *Gleosporium gleosporioide* (36mm), *Colletotrichum musae* (14mm), *Colletortichum capsici* (18mm), *Colletotrichum lamella* (11mm) and *Rhizactonia solani* (12mm) (Table 1). As *Bacillus* spp., were reported to have biocontrol activity, few researchers aimed at *Bacillus* spp. alone (kumari *et al.*, 2017). Many *B. subtilis* strains have been reported for their effective biocontrol activity by producing antifungal compounds (Silo-Suh *et al.*, 1994; Souto *et al.*, 2004; Cazorla *et al.*, 2007; Islam *et al.*, 2012 and Mardanova *et al.*, 2017).

Fig. 2: Isolated potent bacteria from marine sediment sample

It is known that *B. subtilis* species are heterogeneous both phenotypically and genotypically (Zhao *et al.*, 2014). High genetic heterogeneity of different *Bacillus* species (Choudhary and Johri, 2009 and Kopac *et al.*, 2014) particularly *B. subtilis* allows to suggest that search and identification of new strains from different sources may expand the number of practically important strains and to improve our understanding of mechanisms involved in antagonistic interactions. Antifungal action of *Bacillus* metabolites may be due to disruption of fungal cell wall and inhibition of normal conidia development (Fujiwara *et al.*, 2013).

Table 1: Antagonistic activity of Isolate MS21 from marine sediment sample against plant fungal pathogens

Plant fungal pathogens	Zone of clearance (mm)
Macrophomina phaseolina	27
Sclerotium roysii	16
Phytophthora infestans	28
Aspergillus niger	34
Alternaria alternata	28
Gleosporium gleosporioide	36
Colletotrichum musae	14
Colletortichum capsici	18
Colletotrichum lamella	11
Rhizactonia solani	12

The genus *Fusarium* includes plant pathogenic spp. such as *F. avenaceum, F. culmorum, F.equiseti, F.graminearum, F.oxysporum, F.sporotrichoides, F.verticillioides*. When acting as pathogens they mainly attack immature host plants and causes seedling blight, root-crown and foot-rot can penetrate only in damage tissues such as snow mould, leaf and stem infections. Diseases caused by *Fusarium* are popularly referred to as fusarioses (Toppo and Naik, 2015). Fungi like *Trichoderma,* and bacteria like *Bacillus, Serratia, Alteromonos* were reported to have chitinolytic activity (Elad *et al.,* 1982 and Tsujibo *et al.,* 1998).

Biochemical identification of potential strains

The potential isolate MS21 was identified as *Bacillus subtilis* using biochemical methods as per Bergey's manual of systematic bacteriology and it was designated as *Bacillus subtilis* MS21 (Table 2).

Table 2: Biochemical identification of Isolate MS21 (*Bacillus subtillis* MS21) from marine sediment sample

Test	Result
Gram´s staining	+
Morphology	Rod
Motility	+
Catalase	+
Indole	_
Methyl red	_
Voges proskauer	+
Citrate utilization	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Spore	+
Fermentation test	
Glucose	+
Arabinose	+
Xylose	_
Lactose	+
Sucrose	+
Raffinose	_
Galactose	_
Maltose	_
Manitol	+
Oxidase	+

Growth optimization of the potential strain

In the present study, the physicochemical parameters like incubation period, agitation, pH, temperature, salinity, carbon and nitrogen sources were optimized for the growth of potential bacterial strain. The effects of various physicochemical parameters on growth of *B. subtilis* MS21 showed that 36 hrs of incubation period, 150 rpm agitation, pH- 8.0, temperature-35^{o}C, salinity-1.0%, 2% sucrose as carbon source and 1% beef extract as nitrogen source were found to be the ideal conditions resulted in the maximum growth (Figs. 3-11).

The present study was in agreement with Nalisha *et al.*, 2006 who observed maximum growth of *B. subtilis* at 36 hrs of incubation as in the present study (Fig.3). Okanlawon *et al.*, 2010 found highest growth at 48 hrs for most of the isolates in their study. Prescott *et al.*, 2005 and Ynte *et al.*, 2004 observed *B. cereus* was able to grow between 18 to 48 hrs. The shorter incubation period seemed to be ideal for industrial production of biocidal product. As in the present study Li et al., 2009 found150 rpm as the ideal shaking condition for the production of antifungal protein from *Bacillus subtilis* strain B29 (Fig. 4).

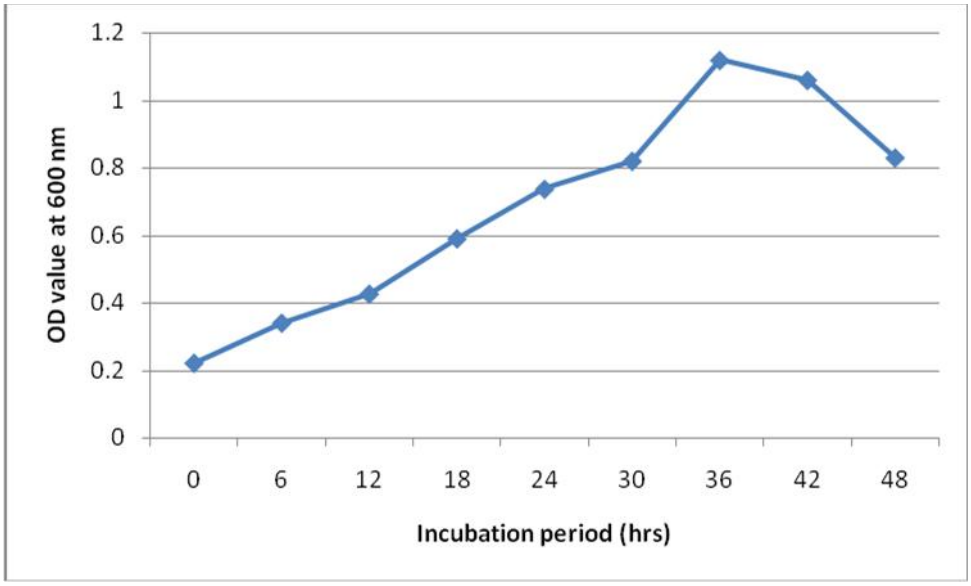

Fig. 3: Effect of incubation period on growth of *Bacillus subtilis* MS21

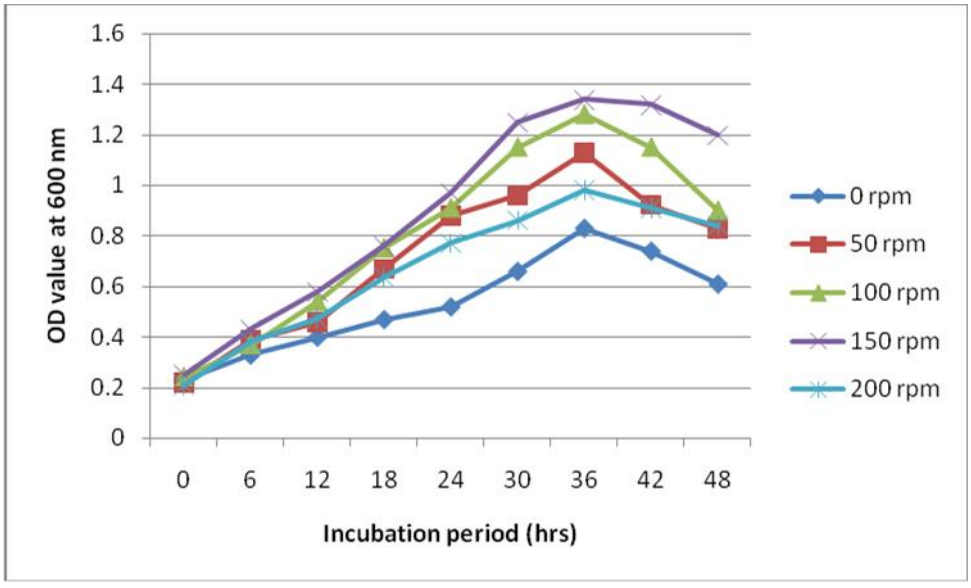

Fig. 4: Effect of static and agitation on growth of *Bacillus subtilis* MS21

Regarding pH Silo-Suh *et al.,* 1994 with *Bacillus cereus* UW85; Okanlawon *et al.,* 2010 with *B. cereus* and Pathak (2011) with *B. subtilis* K1observed maximum growth pH 7; pH 9 and pH 7-9 respectively while Dhinakaran *et al.,* 2012 observed pH 8 as the optimum for the growth of biocontrol protein producing *Corynebacterium sp.* The present study was in agreement with the above findings i.e. maximum growth was observed at pH 8 (Fig.5). Thus the present study along with other reports showed that *Bacillus subtilis* is a robust organism tolerant to a wider pH range.

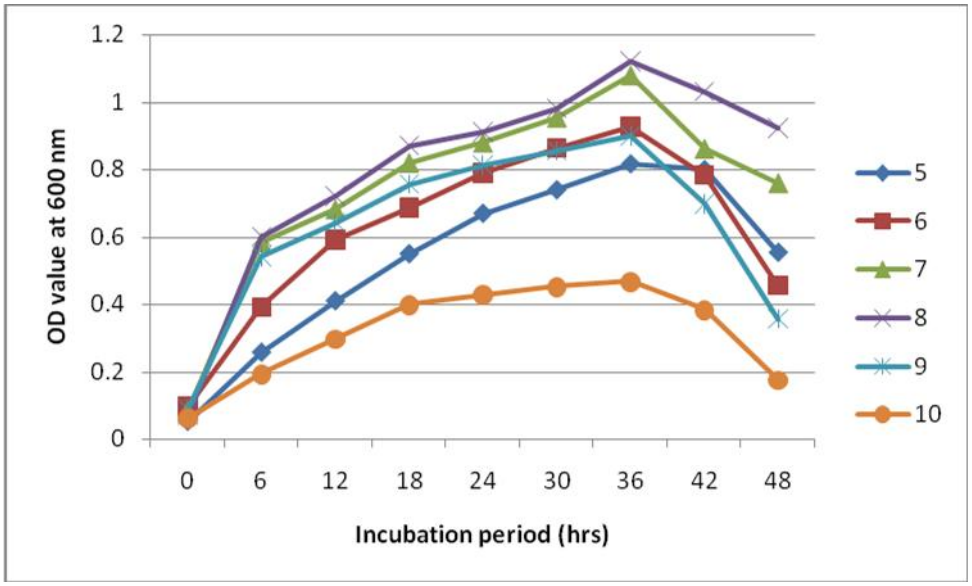

Fig. 5: Effect of pH on growth of *Bacillus subtilis* MS21

In the present investigation, 35°C was found to be ideal for the growth of *B. subtilis* MS21 strain (Fig.6). Hence these bacteria and their products seem to be ideal for the prevailing conditions in most part of the Indian soil. Okanlawon *et al.,* 2010 observed optimum growth of *B.cereus* at 37ºC. Johnson and Snygg (1974) reported that the optimum temperature for *B. cereus* was between 30 and 37°C but some strains could grow at temperature as low as 4.5°C and up to 55°C on the higher side. In *B. Subtilis,* an antagonistic organism to the pathogen of apple crown rot had the optimal temperature around 21-28°C for the production of antifungal compounds. But 25°C was found to be optimum for the growth of the producer strain (Gupta and Utkhede, 1987). Another *B. subtilis* strain, showed optimum temperature for the production of antifungal substance at 30°C in liquid cultivation, but at below 25°C in solid state cultivation (Ohno *et al.,* 1993, 1995).

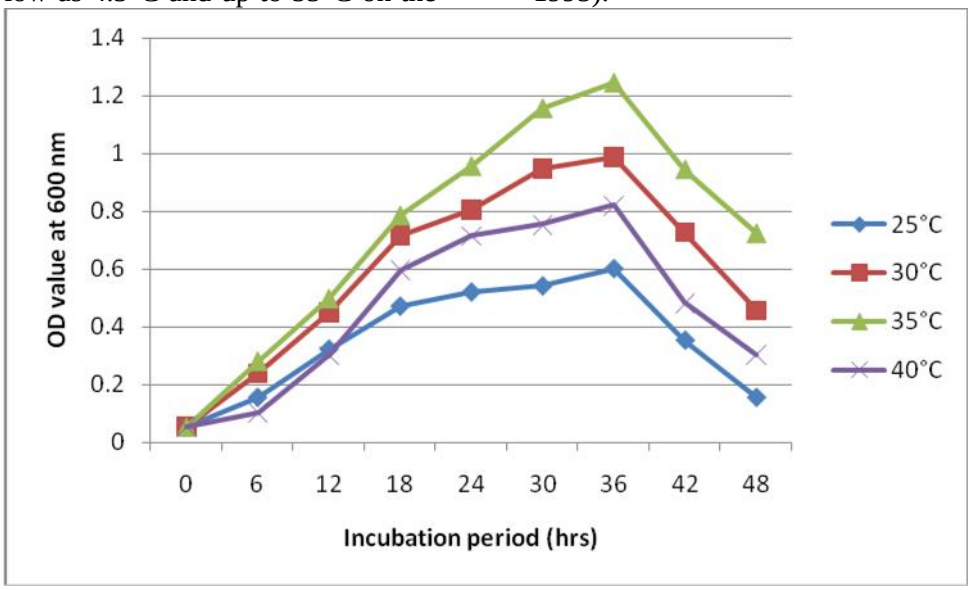

Fig. 6: Effect of temperature on growth of *Bacillus subtilis* MS21

In the present study 1% NaCl supported maximum growth of *B. subtilis* MS21 (Fig.7), whereas Baindara *et al.,* 2013 observed 14% NaCl as the optimum for the growth of halotolerant *Bacillus subtilis* strain SK.DU.4 isolated from a rhizosphere soil. Salinity requirement seemed to be based on the environment from which the strain was originated.

Sucrose (2%) was found to be an ideal carbon source for the growth of *B. subtilis* MS21 in the present work (Figs. 8 and 9). Pathak (2011) and Usama (2003) observed starch and lactose as the ideal carbon sources respectively. Besson *et al.,* 1987 studied the influence of growth media on production of iturin A by *B. subtilis* using glucose as carbon source. Mizumoto *et al.,* 2007 showed addition of glucose as carbon source in minimal salt medium containing Okra enhanced the bioactive iturin A production in solid state fermentation (SSF) by *B. subtilis* RB14-CS. Joshi *et al.,* 2008 observed glucose in minimal salt media enhanced the production of lichenysin (34 g/L) by *B. licheniformis.* Nalisha *et al.,* 2006 observed 1% of oil palm root as the most preferred carbon source. Usama (2003) tested several carbon sources reported that the maximum growth of *B. subtilis* and β-glucanase production was obtained with lactose as sole carbon source. Similarly, in the present, work 1% beef extract was found to be an ideal nitrogen source while Islam *et al.,* 2012 found 1% soytone as the ideal nitrogen source for the growth of antifungal compound producing *Bacillus subtilis* C9 against the plant pathogenic fungi *Rhizoctonia solani* (Figs.10 and 11). Joshi *et al.,* 2008 found ammonium nitrate as a nitrogen source in minimal salt media enhanced the production of lichenysin (1 g/L) in *B. licheniformis*.

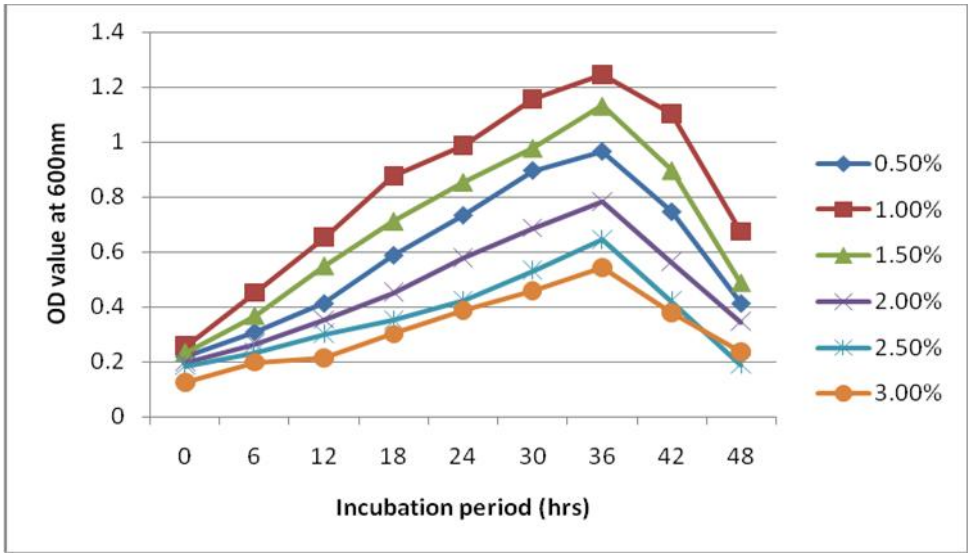

Fig. 7: Effect of NaCl (salinity) on growth of *Bacillus subtilis* MS21

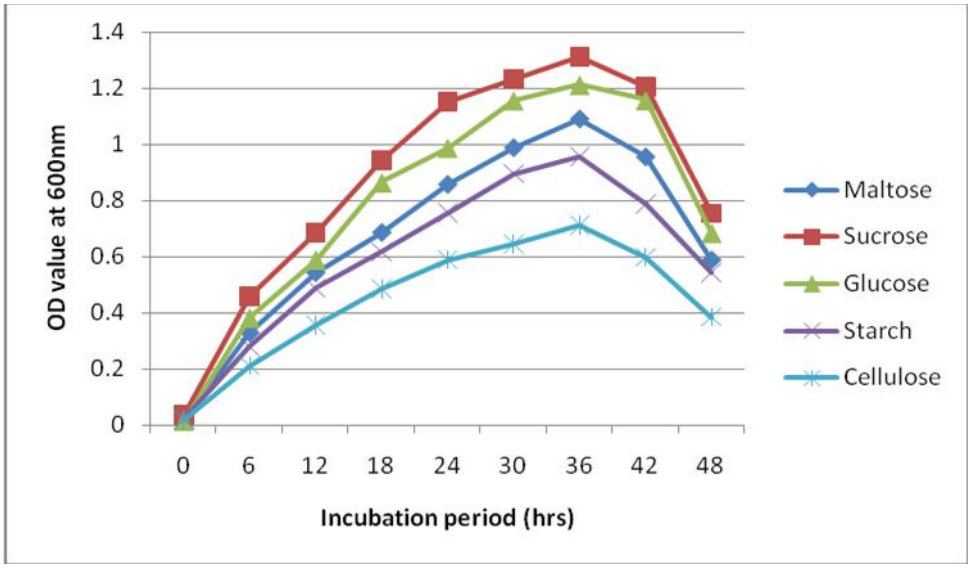

Fig. 8: Effect of carbon sources on growth of *Bacillus subtilis* MS21

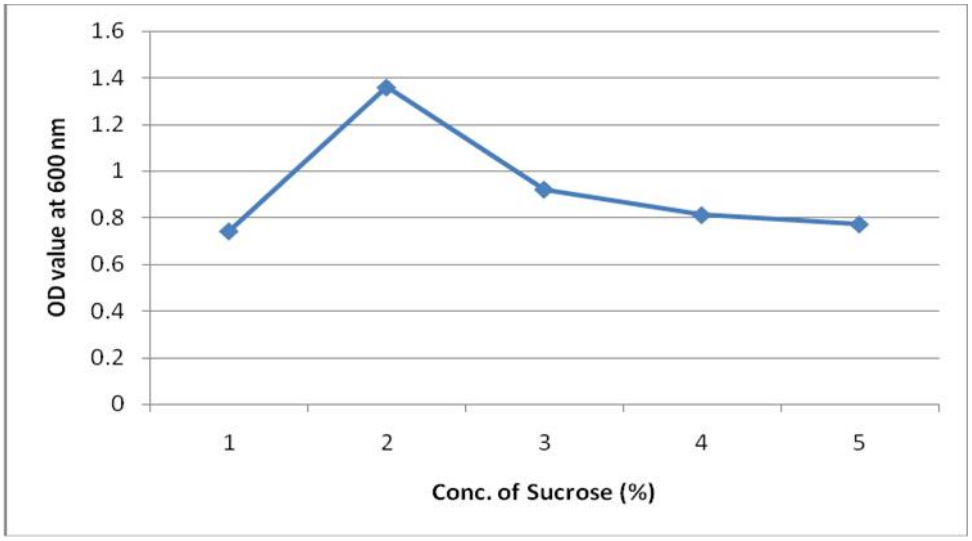

Fig. 9: Effect of concentration of carbon sources on growth of *Bacillus subtilis* MS21

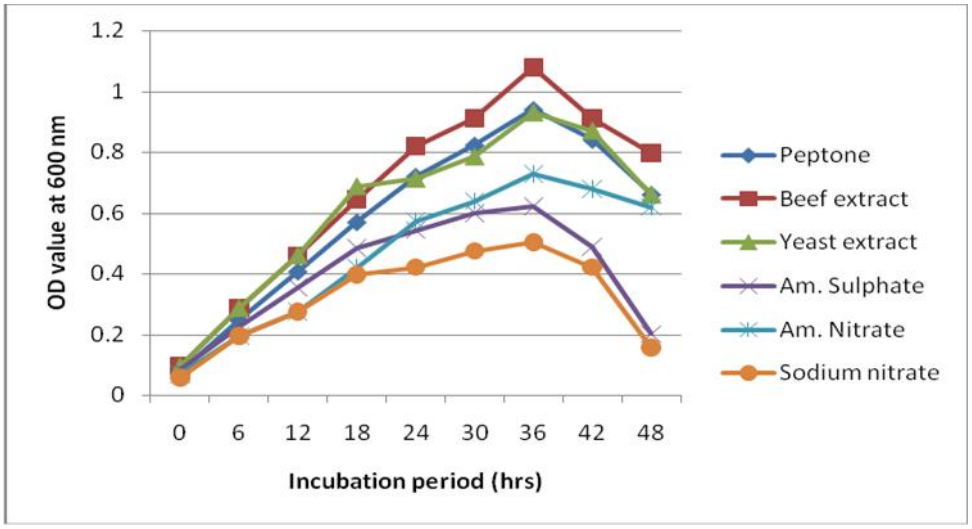

Fig. 10: Effect of nitrogen sources on growth of *Bacillus subtilis* MS21

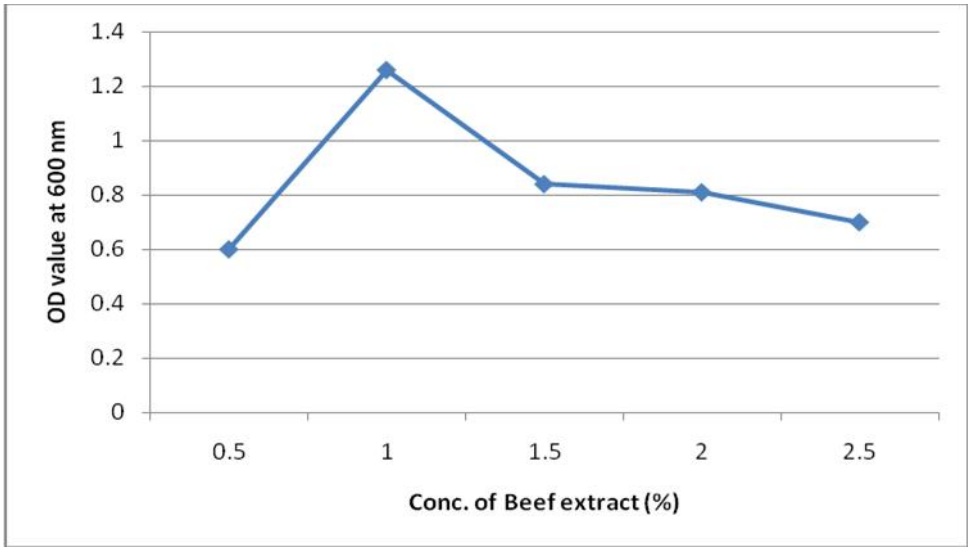

Fig. 11: Effect of concentration of nitrogen sources on growth of *Bacillus subtilis* MS21

Mass cultivation in shake flask

The optimized growth parameters for *Bacillus subtilis* MS21 were used for mass scale cultivation in the shake flask which gave a maximum of 1.92 OD of growth. Phae and Shoda (1991) used submerged fermentation to produce an antagonistic lipopeptide from *B. subtilis.* Mass scale followed by purification resulted in a protein which showed an enhanced activity against most of the fungal pathogens tested.

Conclusion

Thus the present investigation on isolation, screening and growth optimization of a potential biocidal bacteria from sediment sample collected from Thengapattanam estuary was a worth try as the strain *B. subtilis* MS21 showed biocontrolling antagonistic activity against wide range of plant fungal pathogens tested and hence can be used as an effective biocidal agent in combating plant fungal diseases

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