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# Anti-Oxidant and Enzyme-Inhibitory Potential of Marine *Streptomyces*

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## ABSTRACT

Marine actinomycetes are potential source for the discovery of novel compounds and enzymes. Though extensive research on marine actinomycetes is underway globally, the actinomycetes research from Indian marine ecosystem is unexplored and understudied. Hence, the present research is focussed on the screening of bioactive compounds from marine actinomycetes isolated from Indian coastal region. This study is designed to determine the antioxidant and enzyme inhibitory potential of *Streptomyces* sp. VITMSS05 strain, isolated from Marakkanam, southern coast of India. An actinomycetes strain designated as VITMSS05 was isolated. This strain was cultivated in Starch Caesin Agar medium (SCA) supplemented with sea water. The cultural, morphological and molecular characterization was determined for the isolate. The crude extract of the isolate was extracted with ethyl acetate. Antioxidant activity of the crude extract was determined by DPPH radical scavenging assay. Alpha amylase and alpha glucosidase inhibitory potential of the extract was determined. Based on the phenotypic and phylogenetic analysis the strain was identified as *Streptomyces* sp. Significant antioxidant activity of the extract was observed with an IC50 value of 92.49 μg $mL^{-1}$. The extract shows 64.1% inhibition on α-amylase and 91.5% inhibition on α-glucosidase at 100 μg $mL^{-1}$ with an IC50 value of 385.97 and 42.89 μg $mL^{-1}$. From the results it is evident that the ethyl acetate extract of *Streptomyces* sp. VITMSS05 has potent antioxidant and enzyme inhibitory activity *in vitro*. The combined effect of free radical scavenging and enzyme inhibition makes it a potent anti diabetic drug.

**Keywords:** Anti Oxidant, α-Amylase, α-Glucosidase, *Streptomyces* sp. VITMSS05

## 1. INTRODUCTION

It has been estimated that 285 million people, worldwide, have diabetes and there will be a 54% increase by 2030 (Shaw *et al*., 2010). Various pharmacological approaches have been used to treat diabetes; one of the most beneficial therapies is to reduce the post prandial glycemia after the meal (Kim *et al*., 2005). The absorption of glucose can be retarded by inhibiting the carbohydrate hydrolysing enzymes (Kim *et al*., 2005) thereby resulting in decrease in postprandial hyperglycemia. The breakdown of starch to maltose and maltose to glucose was carried out by α-Amylase and the released glucose will be utilized by the body (Kotowaroo *et al*., 2006). α-glucosidase catalyzes the final step in the digestive process of carbohydrates. Its inhibitors can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia and could be useful for treating diabetic and obese patients (Toeller, 1994). Enzyme inhibitors are now receiving increased attention, not only for studying the enzyme structure and reaction mechanism but also for

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pharmacological (Bode and Huber, 1992) and agricultural applications (Terashita *et al*., 1980). Alpha amylase and alpha glucosidase inhibitors such as acarbose, valiolamine, trestatin and amylostatin were isolated from microorganisms (Remi and Jean, 2004) and have been used to control the diabetes. *Streptomyces* is a proven source of microbial enzyme inhibitors (Umezawa, 1972).

Apart from enzyme inhibitors free radicals also responsible for type II diabetes. Free radicals are the product of normal metabolism but oxygen metabolites are toxic (Halliwell and Gutteridge, 1989; Yu, 1994) and activate nuclear factor-κB resulting in upregulation of interleukin-1, interleukin-8 and tumor necrosis factor (Grimble, 1994). They cause oxidative damage to biomolecules such as proteins, lipids, lipoproteins and DNA (Shetgiri and Mello, 2003; Gopinathan *et al*., 2004). Several enzymes and radical scavengers (Nasik, 2003) possessed by our body which constitute the repair systems for biomolecules are damaged by free radicals (Gopinathan *et al*., 2004). Antioxidants can either inhibit or delay the oxidation of the substrate in a chain reaction and therefore appear to be important (Halliwell *et al*., 1992). Previous research reports suggest that actinomycetes are potential producers of antioxidant compounds (Isik *et al*., 2006).

Marine Actinomycetes have emerged as a rich source of novel compounds. Actinomycetes are potent source for the production of antibiotics and other secondary metabolites. Each strain has the potential to produce 10-20 metabolites (Bently *et al*., 2002; Sosio *et al*., 2000). The marine environment exhibits different characteristics when compared to the terrestrial environment and they have a potential for new enzymeinhibitors and antioxidants. Reports states that actinomycetes are powerful producers of antioxidants and enzyme inhibitors (Bull *et al*., 2000). The objective of this study is to investigate the antioxidant and enzyme inhibitory activity of marine *Streptomyces* isolated from marakkanam salt pan.

## 2. MATERIALS AND METHODS

### 2.1. Sampling and Isolation

The soil samples were collected from the depth of 2 feet from Marakkanam (Latitude (N) 13°15, Longitude (E) 80°21′) coast, India. The samples were transported to the laboratory aseptically. The samples were dried in room temperature and used for isolation. Serial dilution of the sample was done and aliquots of each dilution were plated on to starch casein agar plates. The media was prepared with 25% sea water and 25% soil extract (Prepared by mixing the air dried soil sample and water and then filtered) and the growth media was supplemented with antibiotics such as Amphotericin-B (25 μg $mL^{-1}$) and streptomycin sulphate (25μg $mL^{-1}$) (Himedia, Mumbai, India). The plates were incubated at room temperature for 7-15 days. The strains were subcultured regularly inorder to keep them viable for longer period.

### 2.2. Media and Cultural Condition Optimization

The cultural conditions were determined by inoculating the isolate in various media (SCA, ISP1, ISP2, ISP3 and AIA) and the growth was investigated. The effects of cultural conditions like different incubation temperatures (15, 30, 37 and 45°C), different pH (3, 5, 6, 7 and 9) and NaCl concentrations (1, 3, 5, 7 and 9) on the growth of the isolate were studied by measuring the dry mycelial weight.

### 2.3. Taxonomy

The morphological, cultural, physiological and biochemical characterization of the isolates were carried out as described in International *Streptomyces* Project (ISP). The morphology was observed at light microscope and the substrate mycelium was further analyzed by scanning electron microscopy (Hitachi S4000). The Hipura bacterial DNA isolation and purification kit (Himedia, India) was used for the isolation of DNA and amplified by PCR using a master kit and Medoxmix (Medox, India). Universal 16S rRNA primers were used (forward primer FC27 and reverse primer RC 1492) for the amplification of 16S rDNA. Using the earlier reports (Mincer *et al*., 2002; Magarvey *et al*., 2004) the methodology for sequencing was adapted. An NCBI BLAST search was performed and a phylogenetic tree was generated using the neighbour joining method (Saito and Nei, 1987). MEGA version 4 software was used to display the phylogenetic tree.

### 2.4. Metabolite Extraction

The strain was inoculated into SCA broth supplemented with a pH of 7.2 and incubated for 7 days in a rotary shaker (110 rpm) at 28°C. The growth was checked every day. After seven days of incubation the broth was collected and centrifuged at 10000 rpm for 10 min at 10°C. The supernatant was separated from the pellet. The supernatant was extracted twice with ethyl acetate and the extract was concentrated in rotary vaccum and the lyophilized extract was used to carry out the assays. α-Amylase inhibition assay: The α-amylase inhibitory assay was carried out as described earlier

(Suthindhiran *et al*., 2009). α-Amylase solution (0.5 mg $mL^{-1}$) was prepared in 0.02M Sodium phosphate buffer. The crude extracts with different concentration were taken. About 500µl of the extract was added to 500 µL of the enzyme solution and incubated for10min at 25°C. 1% starch solution prepared in 0.02 M Sodium phosphate buffer was added to each tube and incubated at 25°C for 10 min. About 1 mL of dinitrosalicylic acid was added to stop the reaction. The test tubes were incubated in boiling water bath for 5min and then cooled to room temperature. The reaction mixture was then diluted by adding 10 mL of distilled water and the absorbance was measured at 540 nm. The percentage of inhibition was calculated by:

% inhibition = [(A540 control-A540 extract)]
X 100/A540 control

α-Glucosidasde inhibition assay: The α-glucosidase inhibitory assay was carried out as described earlier (Suthindhiran *et al*., 2009). About 50µL of extract (different concentrations) was added to 100µL of α-glucosidase solution (1.0 U/mL) prepared with 0.1M phosphate buffer (pH-6.9) were incubated in 96 well plates at 25°C for 10 min. Then 50 µL of 5Mm p-nitrophenyl α-D-glucopyranoside solution in 0.1M phosphate (pH- 6.9) was added to each well. After incubation the absorbance at 405 nm was read at micro array reader and compared to the control which had 50 µL of buffer solution in place of the extract. The percentage of inhibition was calculated by:

%inhibition = [(control405-extract405)]
X 100/control540

## 2.5. Antioxidant Activity

The antioxidant activity was carried out as described earlier (Yang *et al*., 2006). The concentrations of extracts and DPPH were 1mg $mL^{-1}$ and 0.002% respectively. 2 mL of DPPH solution was mixed with 2 mL of extract. The reaction mixture was incubated in dark for 30 min. The optical density was measured at 517 nm using UV-Vis Spectrophotometer. The scavenging activity of the extract against the stable DPPH was calculated using the following equation:

Scavenging activity (%) = A-B/A x 100

where, A is the absorbance of DPPH solution and B was the absorbance of DPPH solution with extract.

## 2.6. Statistical Analysis

All analysis were carried out in triplicate and the data were expressed as mean ± SE (standard error).

# 3. RESULTS

The cultural, morphological, biochemical and physiological characteristics reveals that the strain VITMSS05 belongs to the genus *Streptomyces*. The isolated organism is gram positive and non-motile. The aerial mycelium is branched with long spore chains (**Fig. 1**). The colonies were white to grey in colour instarch casein agar medium. The spore surface is smooth when observed at 10µm under scanning electron microscope. Diffusible pigment and melanin pigment were not been produced by the strain. The strain shows abundant growth in Starch casein agar medium and the strain grows well when cultivated at 28°C at pH 7.4.

The strain requires 3% NaCl for optimal growth. Among carbon sources the strain shows abundant growth in fructose, galactose and lactose but failed to grow in maltose, mannitol and xylulose. The effect of pH, Temperature and salt concentration on the growth of the isolate is given in the **Table 1**. The strain shows good growth in pH 6, 7 and 9, while it shows moderate growth in pH 5. The strain grows at temperatures 28, 37°C and the optimum temperature was found to be 28°C.

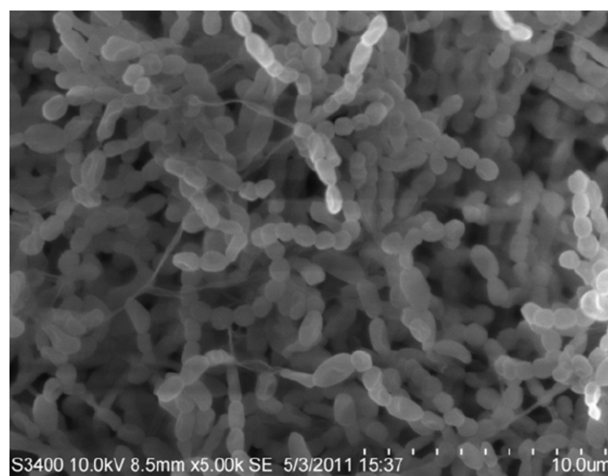


**Fig. 1.** Scanning electron micrograph of VITMSS05 grown in optimized medium at 28oC for 5 days. (The bar represents 5 µm)

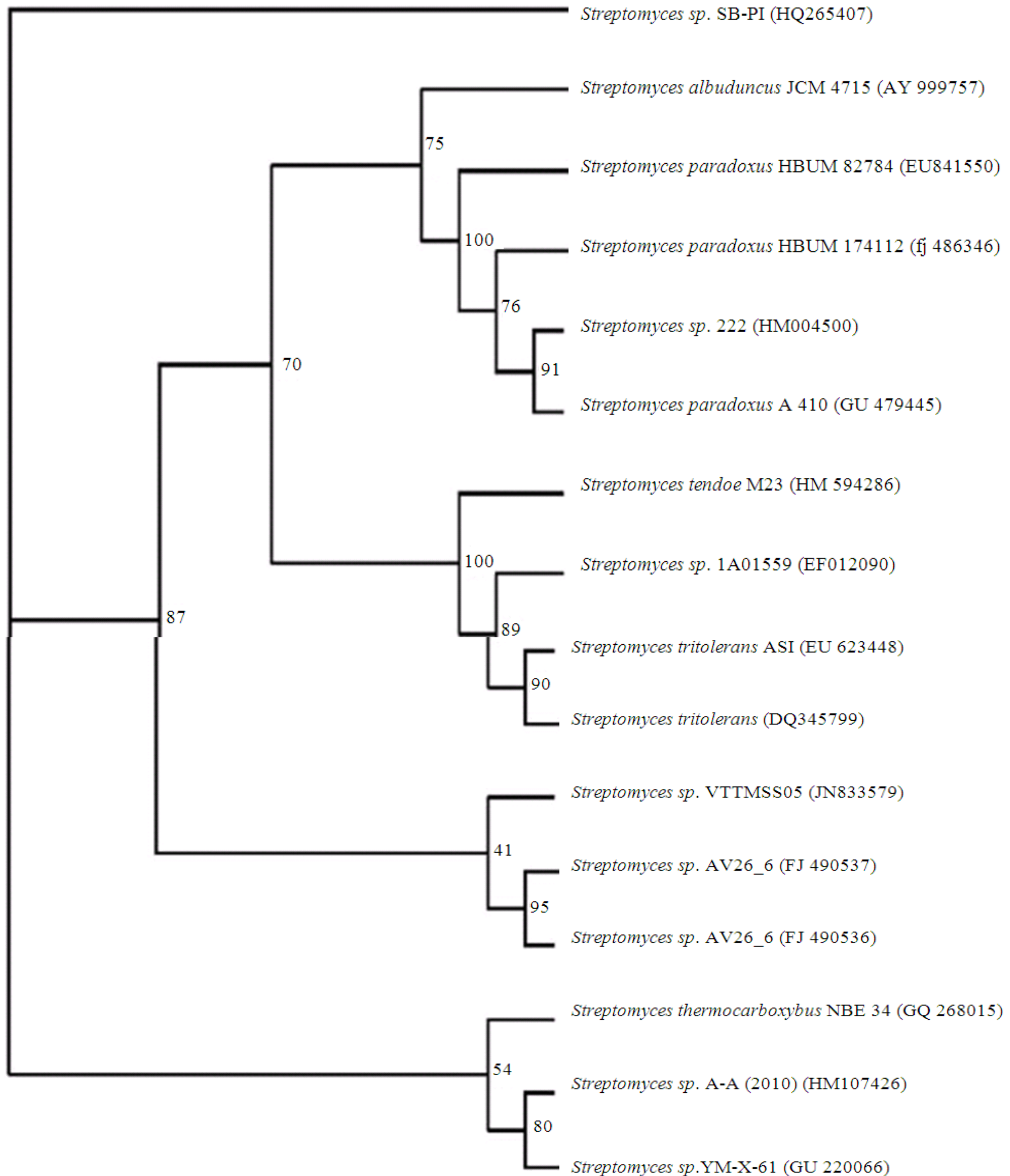


**Fig. 2.** The phylogram showing the position of strain VITMSS05 with other *Streptomyces* based on 16S rRNA partial gene sequence

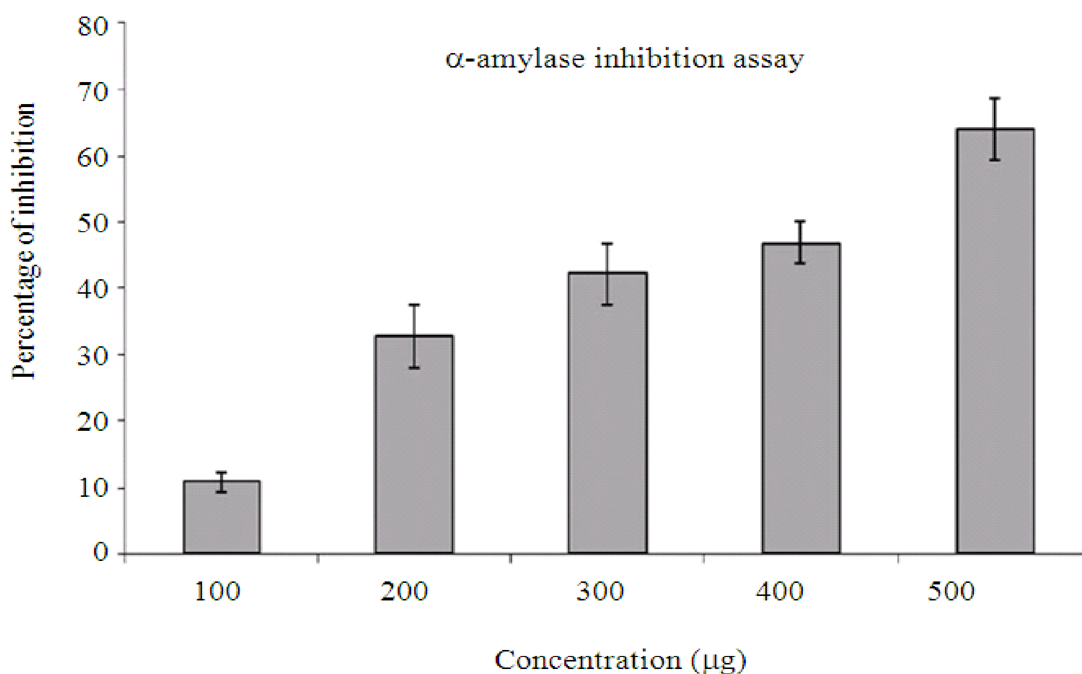


**Fig. 3.** DPPH (1, 1- diphenyl 2-picryl hydrazyl) scavenging activity of compound extracted from *Sterptomyces sp*. VITMSS05. The values are mean ± SD

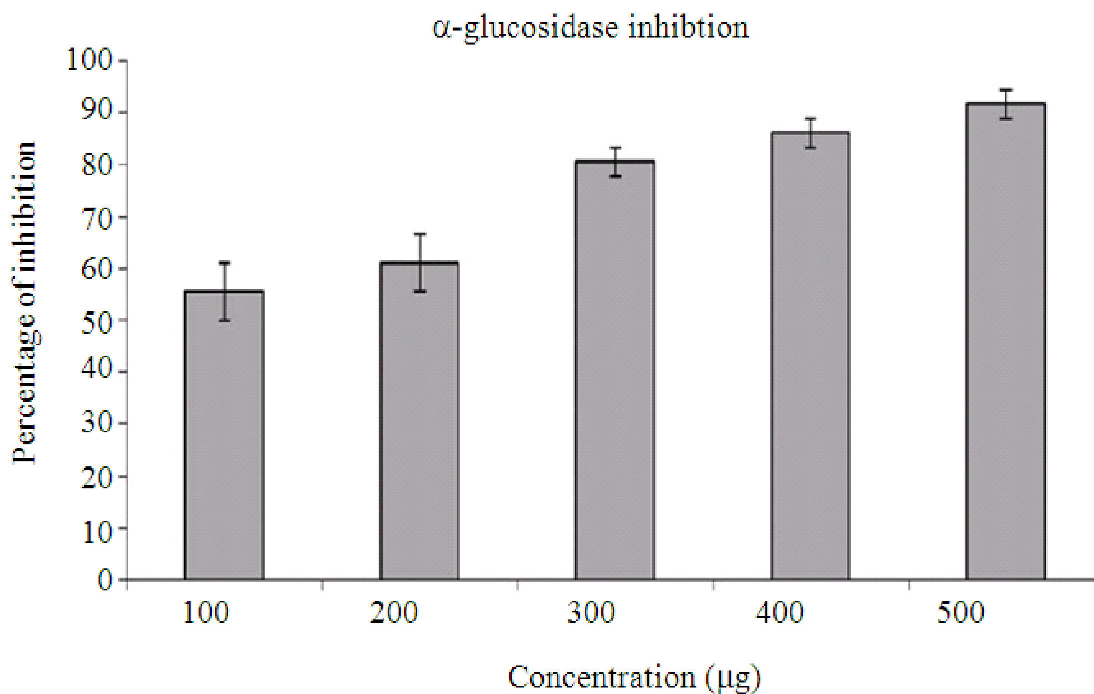


**Fig. 4.** α-Amylase inhibitory potential of *Sterptomyces sp*. VITMSS05. The values are mean ± SD

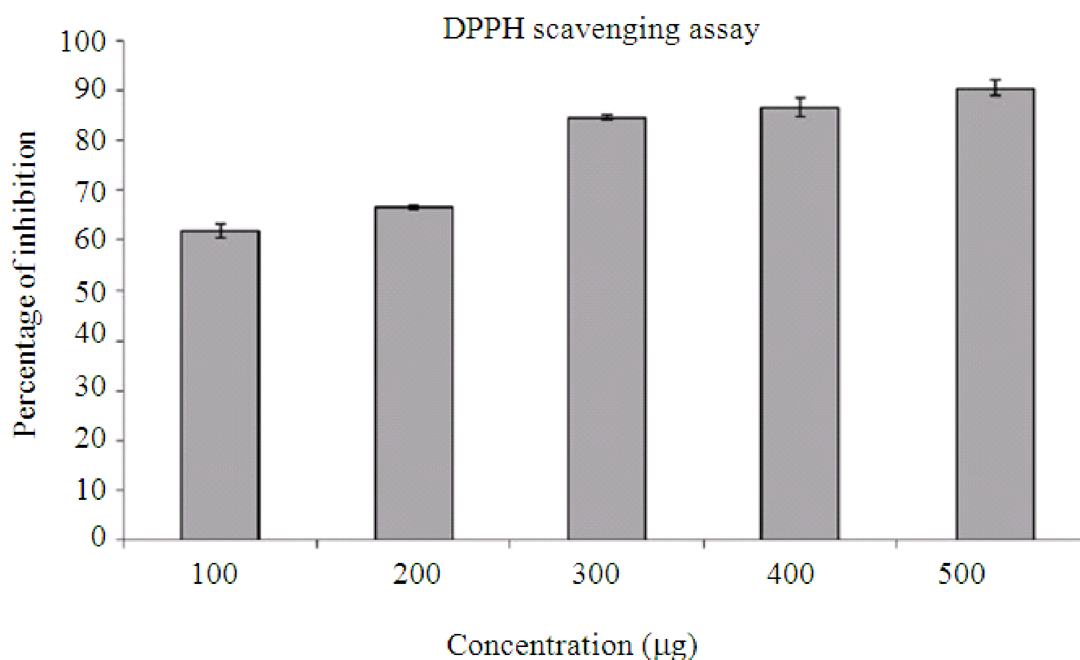


**Fig. 5.** α-Glucosidase inhibitory activity of *Streptomyces sp*. VITMSS05. The values are mean ± SD

### 3.1. Molecular Taxonomy

NCBI BLAST search analysis showed that the sequence was 99% similar with that of *Streptomyces tritolerans*. A neighbour joining tree based on 16S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces* family. A phylogenetic tree was constructed based on kimura method, also showed distinct position of the isolate (**Fig. 2**). Based on the molecular taxonomy and phylogeny the strain was identified as *Streptomyces* and designated as *Streptomyces* sp. VITMSS05. The 16S rRNA sequences were submitted to the GenBank under the accession number JN833579.

### 3.2. Alpha Amylase and Alpha Glucosidase Inhibition

The *In vitro* Alpha amylase inhibitory activity demonstrates that the ethyl acetate extract of the strain VITMSS05 has inhibitory activity. Significant inhibition exhibited by the ethyl acetate extract at a concentration of 500 µg $mL^{-1}$ with 61.1% inhibition. The $IC_{50}$ value for alpha amylase inhibition is 385.97 µg $mL^{-1}$. Activity increases with increase in the concentration (**Fig. 3**). The *In vitro* alpha glucosidase activity of the ethyl acetate extract of the strain VITMSS05 is given in the **Fig. 3**. Analysis of the data confirms that maximum inhibition (91.5%) was seen at 500 µg $mL^{-1}$ (**Fig. 4**). The IC50 value for alpha glucosidase inhibition is 42.89 µg $mL^{-1}$.

### 3.3. Antioxidant Assay

The results of the DPPH scavenging activity of VITMSS05 extract was shown in the **Fig. 5**. In this study the ethyl acetate extract of the strain VITMSS05 showed 90.57% activity at 500 µg $mL^{-1}$ concentration. The IC50 value of the extract is 92.49 µg $mL^{-1}$. The antioxidant activity was found to be dose dependent.

## 4. DISCUSSION

Marine micro organisms are known to produce various novel metabolites. Among microorganisms, Actinomycetes are the largest producer of antibiotics (Lazzarini *et al*., 2000) and other novel metabolites. These actinobacteria produces resistant spores and are salt tolerant (Okazaki and Okami, 1975). In the present study we have isolated *Streptomyces* sp. VITMSS05 from Marakkanam salt pan. Comparison of 16S rRNA sequences of the strain VITMSS05 with the corresponding sequences confirmed that VITMSSO5 belonged to the genus *Streptomyces*.

**Table 1.** Effect of pH, Temperature and Salt concentration on the growth of VITMSS05

| Different parameters | Results |
|---|---|
| **Effect of Temperature** | |
| 15°C | No growth |
| 28°C | Abundant growth |
| 37°C | moderate growth |
| 45°C | moderate growth |
| **Effect of pH** | |
| 5 | Moderate growth |
| 6 | Good growth |
| 7 | Good growth |
| 9 | Good growth |
| **Effect of NaCl** | |
| 1% | Good growth |
| 3% | Good growth |
| 5% | Moderate growth |
| 7% | No growth |
| **Nitrogen sources** | |
| Citrate | Positive |
| Nitrate | Negative |
| Urease | Positive |
| Gelatin | Negative |
| Carbon | sources |
| Maltose | No growth |
| Mannitol | No growth |
| Xylulose | No growth |
| Galactose | Good growth |
| Lactose | Good Growth |
| Fructose | Good growth |

The strain shows 99% similarity with *Streptomyces tritolerans* ASI which has been isolated from earthworm gut and shows activity against plant pathogenic bacteria and fungi. *Streptomyces* AV26_2 shows 99% similarity with our strain and was isolated from leaf cutting ants, this strain produces candicidin macrolide and shows antifungal activity. Xylosidase enzyme producing strain *Streptomyces* sp. YMX-6, shows 99% similarity with our strain. The substrate mycelium is highly branched and the hyphae differentiated into long chain spores. The strain does not produce any soluble pigments. Morphological and cultural characteristics indicate that the strain VITMSS05 can be assigned to the genus *Streptomyces*. The strain utilizes galactose, fructose and lactose. The culture was optimized with varying media, salt and carbon sources (**Table 1**) to enrich the yield of metabolite.

The strain was found to be moderately halophilic as it grows at salt concentrations ranging from 5-20%. We have already isolated many strains of actinomycetes which were found to have several bioactivities (Suthindhiran and Kannabiran, 2009a; 2009b; Suthindhiran *et al*., 2011; Suthindhiran and Kannabiran, 2010).

About 90-95% of patients have type II diabetes and the treatment for type II diabetes has many limitations (Mark and Grell, 1997). α-amylase and α-glucosidase are the major enzymes involved in type II diabetes. Inhibitors of these enzymes will inhibit the glucose liberation from carbohydrates and delay the absorption of glucose which will result in postprandial hyperglycemia (El-Ashry, 2000; Franco *et al*., 2002; Jayasri *et al*., 2009). So metabolites from marine origin can be used to treat II diabetes. Recently, *Micromonospora* sp. VITSDK3 was reported for its efficient production of α-amylase and α-glucosidase inhibitors (Suthindhiran *et al*., 2009). In our study, the extract showed 64.1% inhibition (**Fig. 3**) of α-Amylase enzyme and 91.5% inhibition (**Fig. 4**) of α-glucosidase at a concentration of 500 μg $mL^{-1}$. We have focussed on both the aspect of inhibiting the enzymes responsible for diabetes and also the free radical scavenging ability. Free radicals are highly unstable which cause damage to other molecules and attain stability by extracting electrons from them (Ali *et al*., 2009). Natural antioxidants are associated with health benefits (Ali *et al*., 2009). Dietary antioxidants inhibit peroxidation chain reactions and have a protective effect against diabetes development (Feskens *et al*., 1995). Previous reports states that *Streptomyces* metabolise the compounds with antioxidant activity such as isoflavonoids (Komiyama *et al*., 1989), diphenazithionin (Hosoya *et al*., 1996), dihydroherbimycin A (Chang and Kim, 2007), polysaccharide (He *et al*., 2008) and protocatechualdehyde (Kim *et al*., 2008). The ethyl acetate extract of our isolated strain showed 90.57% ofradical scavenging activity. The findings of the present study clarify that enzyme inhibitors and antioxidants were present in the compound which can be used to treat diseases. Further, the chemistry and mechanism of these compounds need to be investigated.

## 5. CONCLUSION

In this study the ethyl acetate extract of *Streptomyces sp*. shows significant inhibition against porcine pancreatic amylase and yeast glucosidase and also has the potential to scavenge the free radicals. Further structural and invivo studies of these compounds will be helpful for the development of new drug for the treatment of diabetes.

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