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Bioprospecting Studies of Halophilic Bacteria - *Streptomyces* sp MA05 and *Halobacterium* sp MA06

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Abstract: In this study, two halophiles (*Halobacterium* sp MA06 and *Streptomyces* sp MA05) isolated from the salt lake of Chennai were exploited. Optimization of growth condition for both the organisms was done and then subjected for bioprospecting analysis for the enzyme (amylase) and pigment production. One of the isolates, i.e., *Streptomyces* sp MA05, was found to produce amylase. Thus that organism was subjected to amylase production, and the produced amylase was characterized. The other isolate (*Halobacterium* sp MA06) was found to produce orange color pigment, and the pigment was characterized by GC-MS analysis and subjected to optimization of pigment production. The extract of *Streptomyces* sp MA05 and pigment of *Halobacterium* sp MA06 were subjected to antimicrobial and anticancer activity. Only the metabolite of *Streptomyces* sp MA05 had antibacterial activity.

Keywords: Bioprospecting; amylase production; pigment production.

1. Introduction

Bioprospecting organisms from any source are very important, as the obtained products have industrial and medicinal applications [1,2]. Marine organisms have been showing more bioactivities [3,4]. Hypersaline environments such as the sea, lake, estuarine have a notable number of halophilic bacteria and halotolerant bacteria [5,6]. In recent decades, microbes isolated from these hypersaline environments and surroundings are highly explored for many products [7,8]. These halophilic prokaryotes are classified on the basis of the salt requirement for survival and growth as follows - slightly halophiles (1 – 3 % salt), moderate halophiles (3 – 15 % salt), and extremely halophiles (15 – 32 % salt) [9,10]. These halophiles are widespread and abundant in salt lakes, sea, etc. Since they live in extreme conditions, they could synthesize several metabolites and produce useful enzymes, including amylase, esterase, cellulase, protease, etc. [11-14]. These organisms also have a vital role in heavy oil degradation [15], biofuel production, and polyaromatic hydrocarbon production [16]. Metabolites of halophilic isolates from invertebrates were reported to be efficient against various pathogenic bacteria [17-19]. Halophilic *Vibrio* sp. A1SM3–36-8, was reported to have good antibacterial and

anticancer activity [20]. Pigments derived from halophilic bacteria like β carotene and bacteriorhodopsin have shown many applications in optical mechanics [21-23]. Having known the potency of these halophiles, this study was done to optimize the growth condition of two halophiles isolated from Salt lake, Chennai. One of the organisms was producing orange pigment, and the other was a chalky white colony that was producing amylase enzymes. Pigment and the extract of the organisms were tested for various bioactivity studies.

2. Materials and Methods

2.1. Chemicals used and selection of halophilic microorganisms.

All the chemicals were bought from HiMedia, India. Seawater was collected from Kovalam, Chennai, Tamil Nadu, India. Two organisms isolated from the water sample collected from the Salt Lake, Muttukadu, Chennai were used in this study. One was orange pigment-producing organism – *Halobacterium* sp MA06 (Genbank Accession no. MN626450), and the other was chalky white colony-forming organism – *Streptomyces* sp MA05 (Genbank Accession no. MN629934).

2.2. Optimization study.

2.2.1. Growth optimization.

Both the organisms were subjected to grow on halophilic isolation medium (For 1000 mL: Casein powder – 1 g, peptone - 0.5 g, Trisodium citrate – 0.3 g, Yeast extract – 1 g, Potassium chloride – 0.2 g, Magnesium sulphate – 2.5 g, Sodium chloride – 25 g), Seawater medium (seawater + 2% Agar), Seawater with 1 % yeast extract + 1 % peptone + 2 % Agar, Seawater with 1% yeast extract + 1% peptone + 1 % trisodium citrate + 1% KCl + 1% $MgSO_4$ + 2% Agar. pH was set as 5 in all the mediums. The organism was inoculated onto the medium and incubated for 48 – 72 h. The medium which influenced the growth of the organism was used further. The growth pattern was analyzed by its growth on medium with 2 % agar. The above parameters were even checked by Manikandan et al. (2009) [24]. The medium was further optimized for NaCl concentration. After standardizing the NaCl, the medium was subjected to the standardization of optimal $MgSO_4$ concentration, pH, etc.

2.3. Optimization and characterization of pigment.

2.3.1. Pigment production optimization.

The pigmenting organism was allowed to grow on halophilic isolation medium (with 2% Agar) with varied concentrations of sodium chloride, peptone, and yeast extract.

2.3.2. Extraction of pigment.

Orange pigment organism was grown as stated earlier. Colonies were scraped out of the plate using an inoculation loop and immediately transferred to < 3% NaCl solution. The solution was subjected to centrifugation at 10000 rpm for 30 min [25]. The pellet was weighed, and the wet mass was identified. The cell was lysed by adding the pellet with distilled water (1:50) and kept at 4 °C for 24 h. It was then centrifuged at 10000 rpm for 30 min, and the pellet was collected. Pigment extraction was done by adding 90% acetone in the ratio of 1:5 to the pelletized lysed cells [26]. The supernatant (acetone) containing the pigment was collected by

centrifugation. Acetone was evaporated out in the fume hood, and the pigment weight was measured. The percentage of pigment production was obtained as described [27].

2.3.3. Characterization of pigment.

The pigment was characterized by GC-MS analysis (Clarus 680, Perkin Elmer1). Where the Initial temperature in the oven was 60°C for 2 min, ramp temperature was increasing at 10 °C/min to 300°C with a hold of 6 min. The total run time was 32 min. Injector temperature was 250 °C, with a volume of 1 µL. Flow Rate was set as 1 mL/min, and the carrier gas used was helium, and the column used was Elite-5MS (30.0m, 0.25mmID, 250µm df).

2.4. Amylase production.

2.4.1. Qualitative analysis of amylase production.

Media Composition used was as designated in previous studies [27], where the NaCl concentration alone was increased to 10 g / 100 mL. Isolated colonies were streaked on the plate and incubated for 72 h at room temperature. After incubation, the iodine solution was flooded and analyzed for the formation of a clearance zone.

2.4.2. Optimization of amylase production.

Enzyme production media Composition was as follows where NaCl concentration was added as 100 g / L other chemicals are as follows (g / L) Citric acid - 0.42, Yeast extract - 2.5, $MgCl_2.6H_2O$ - 0.25, $MnCl_2.4H_2O$- $1.0x10^{-2}$, $CaCl_2$ - 2.2 x 10^{-3} , $FeCl_3.6H_2O$ - $2.7x10^{-2}$, $CuCl_2.2H_2O$ - 8.5 x 10^{-4}, Soluble starch – 5 g, pH - 7. 1 mL culture (which was grown in the above-mentioned media) was inoculated to the 100 mL above medium and incubated at 37 ^{0}C. After 48 h of incubation, the media was centrifuged at 10000 rpm, and the supernatant was taken as a crude enzyme source as described [28]. The activity of amylase was done as follows – 500 µL of 1% soluble starch in PBS buffer (pH 6.5) was added with 300 µL crude enzyme source and incubated at 37 ^{0}C for 15 min. Following that, it was added with 1 mL DNS reagent. Using spectrometry, reducing sugars released was assayed, and the enzyme unit was demarcated, as described earlier [29]. Optimization of amylase production was done for substrate concentration 0.2 to 1 g, temperature range 25 – 45 °C, time interval 24 - 120 h, and pH 4 - 8.

2.4.3. Amylase characterization.

The role of pH and temperature on amylase activity was done [30] by subjecting the isolated enzymes for its activity in different pH and temperature. Amylase activity was analyzed, as mentioned earlier in this study, as well as earlier studies [31].

2.5. Antimicrobial susceptibility study.

2.5.1. Extraction of antimicrobial metabolites.

The culture filtrate was added with chloroform (1:1). It was taken in separating funnel and shaken vigorously for 2 – 5 min. Then the solution was allowed to stand; still, the organic phase was collected, and it was evaporated in a fume hood.

2.5.2. Agar well diffusion method.

The antibacterial activity of the above extract and pigment was performed against *Staphylococcus aureus* and *Escherichia coli* [32]. The required concentration of extract/pigment was dissolved in Dimethyl sulfoxide (DMSO) and used for the study. Antibiotic disc - Gentamycin (200 mcg) and amoxicillin (200 mcg) was used a positive control. The plate was incubated at 37 ^{0}C for 24h.

2.6. Anticancer activity – MTT assay.

Vero and Hep G2 cell lines were obtained from NCCS, Pune, which was maintained in MEM with 10% serum. *Streptomyces* sp MA05 extract and pigment were subjected to MTT assay as described by Mosman (1983) [33]. Each cell line was seeded with 10,000 cells onto a 96 well plate and incubated at 37 ^{0}C till it reached the confluency in the CO_2 incubator. Plates were washed with PBS and introduced with different concentrations (0.3125, 0.625, 1.25, and 2.5 µg) of either the *Streptomyces* sp MA05 extract or pigment as triplicates. It was incubated and added with 100 µL MTT (5mg / mL) and kept in the dark for 4 – 5 h, following that 100 µL DMSO was added and read at 570 nm. The mortality percentage was calculated.

2.7. Statistical analysis.

All the experiments had been carried thrice, and all the results are given as mean ± standard deviation.

3. Results and Discussion

3.1. Selection of halophilic microorganism.

Following the isolation procedure for halophilic organisms, an orange pigment-producing *Halobacterium* sp MA06 and rough, chalky white colonies forming *Streptomyces* sp MA05 were isolated from salt lake, Chennai (Figure 1). Preliminary morphological observation and Gram staining reaction revealed that pigmented bacteria were gram-negative rod, while white was gram-positive filamentous.

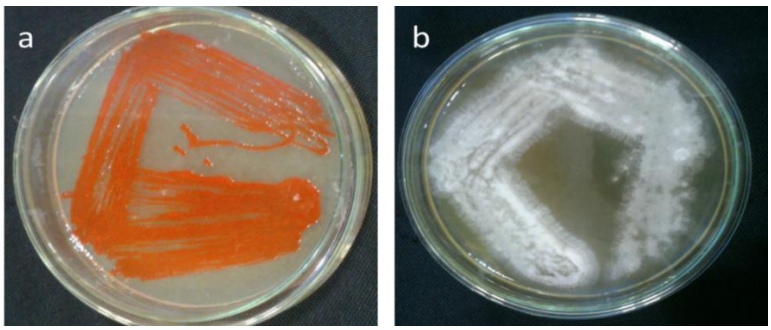

Figure 1. Organisms isolated from salt lake, Chennai a) *Halobacterium* sp MA06. b) *Streptomyces* sp MA06

3.2. Optimization study.

The organisms grew well on the halophilic isolation medium but failed to grow on the rest of the medium (Table 1). This was because of rest of the medium were lacking either nitrogen source or less concentration of NaCl concentration.

Table 1. Growth characteristics of organisms in a different medium.

Complex medium	pH	Bacteria	*Streptomyces sp* MA05
Halophilic isolation medium	5	++	++
Seawater medium	5	N/G	N/G
Sea water + yeast extract + peptone	5	N/G	N/G
Sea water + yeast extract + peptone + tri-Na-citrate +KCl + $MgSO_4$	5	N/G	N/G

++ - good growth, N/G – no growth

Table 2 shows the optimum NaCl concentration for the organism's growth in the halophilic isolation medium. The bacteria were found to grow well at 20 % to 32 % NaCl concentration, whereas *Streptomyces sp* MA05 grew well at 10 %. The pigmented bacterium was found to be extreme halophilic as it grew well on 20 % NaCl, only in the presence of magnesium, and the optimal $MgSO_4$ concentration was found to be 2.5 % (Table 3 and 4). Optimum pH for the pigmented bacteria was found to be 7 and for *Streptomyces sp* MA05 as 5 (Table 5). Our results were on par with Hongyu *et al.* (2009) and Roohi *et al.* (2012), where they found the isolated halophils to grow well at a temperature of 28 – 40 ºC, pH 7.0 – 9.0 and 5 - 20% (w/v) NaCl [34,35]. Halophilic bacteria isolated from a salt mine in Central Anatolia (Turkey) grew best at 3.5 - 4.5 M NaCl concentration and pH 7 - 7.5 [36]. Magnesium has a vital role in the bacteria growth, whereas the *Streptomyces sp* MA05 grew irrespective of magnesium. It was also found that the halophilic bacteria require magnesium, too, for their growth [37]. Mironescu *et al.* (2005) showed the influence of magnesium in halophilic microbe growth [38]. Whereas Alinei *et al.* (2006) also observed the same [39]. The difference is due to the influence of Mg^{2+}, which has limiting action on the microorganisms.

Table 2. Growth characteristics of organisms in Halophilic Isolation Medium with varying NaCl concentration.

NaCl concentration	pH	Bacteria	*Streptomyces sp* MA05
4%	5	N/G	+
10%	5	N/G	+++
16%	5	+	++
20%	5	+++	+
22%	5	+++	+
25%	5	+++	+
32%	5	+++	N/G

+ - presence of growth, ++ - good growth, +++ - abundant growth, N/G – no growth

Table 3. Growth characteristics of organisms in Halophilic Isolation Medium with varying NaCl & $MgSO_4$ concentration.

NaCl	$MgSO_4$	pH	Bacteria	*Streptomyces sp* MA05
20%	-	5	N/G	+
20%	2.5%	5	++	+
10%	-	5	N/G	++
10%	2.5%	5	+	+++

+ - presence of growth, ++ - good growth, +++ - abundant growth, N/G – no growth

Table 4. Growth characteristics of organisms in Halophilic Isolation Medium with varying $MgSO_4$ concentration.

$MgSO_4$ Concentration	pH	Bacteria	*Streptomyces sp* MA05
1.5%	5	+	++
2.5%	5	++	++
3.5 %	5	+	+
4.5%	5	N/G	N/G

+ - presence of growth, ++ - good growth, N/G – no growth

Table 5. Growth characteristics of organisms in Halophilic Isolation Medium at varying pH.

pH	Bacteria	*Streptomyces sp* MA05
4	N/G	N/G
5	++	+++
7	+++	++
8	++	N/G
10	N/G	N/G

+ - presence of growth, ++ - good growth, +++ - abundant growth, N/G – no growth

In our study, the maximum pigment was produced from bacteria in the halophilic agar medium with 32 % NaCl, i.e., 64 % (Figure 2). Further, the maximum pigment (64 %) was produced at medium containing 10 % yeast extract concentration and 15 % peptone concentration (Figures 3 and 4). Pigment production by *Halorubrum sodomense* was reported to be maximum at NaCl concentration of 30% [40]. Salt concentration has a direct influence on pigment production [41].

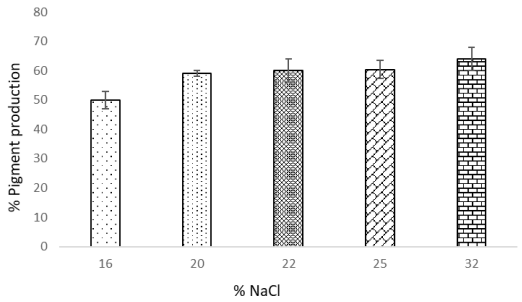

Figure 2. Pigment production in varied NaCl concentration.

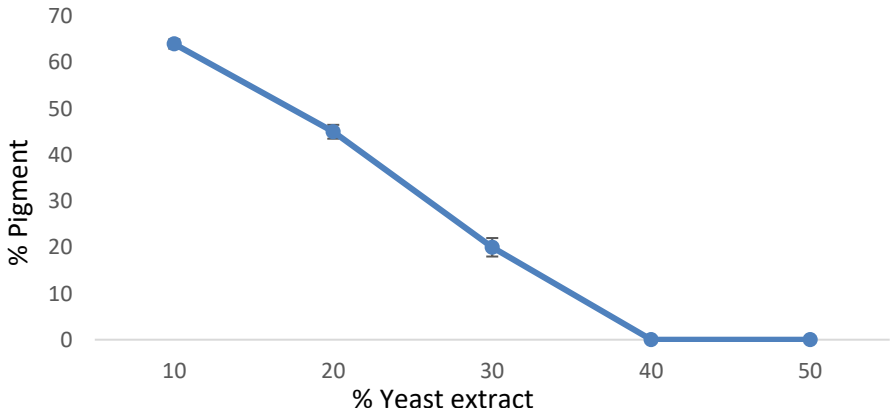

Figure 3. Pigment production in different yeast extract concentration.

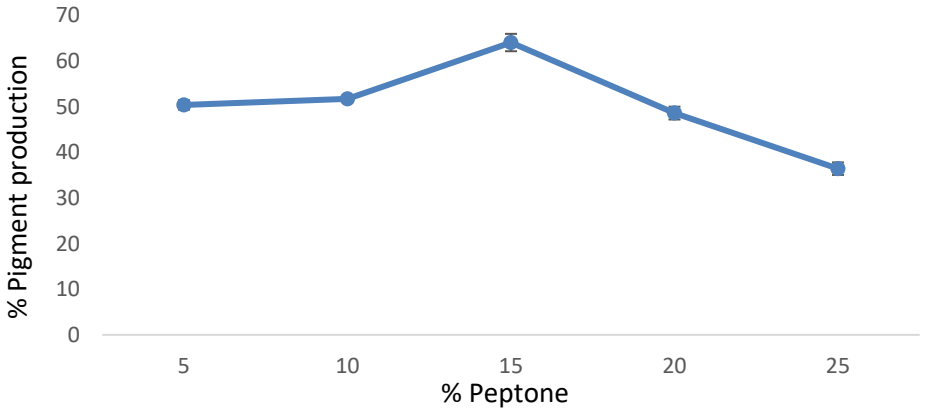

Figure 4. Pigment production in different peptone concentration.

3.3. GC-MS analysis.

The abundant compound shows maximum peak at retention time of 23.06 and 13.3 (Figure 5), which stands for phenol,2,6-bis(1,1dimethylethyl-). Costantino *et al* (1993) found to that 2,6-bis-(1,1-dimethylethyl)phenol derivatives to have anti-inflammatory property [42]. Other compounds like 2-T-Butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid - t-butyl ester (RT - 22.6), methyl 2-hydroxy-eicosanoate (RT -28.5), fumaric acid, 2,4-dimethylpent-3-yl tridecyl ester (RT - 29.6) were also found.

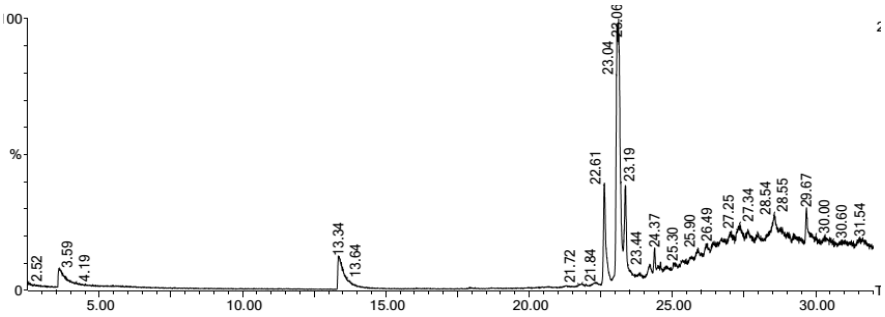

Figure 5. GC-MS analysis of pigment.

3.4. Enzyme optimization.

The optimum amylase was produced at a starch concentration of 0.8 g at 72 h at 40 °C in pH 5 at 10 % NaCl concentration. (Figure 6-9). Amylase production was nil in the absence of sodium chloride; this indicated the halophilic nature of the enzyme production and activity. Similar behavior has been described by Deutch (2002) [43]. Marine *Streptomyces* sp. D1 was reported to produce amylase in a temperature range between 37 and 55 ^{0}C in 7 days [44].

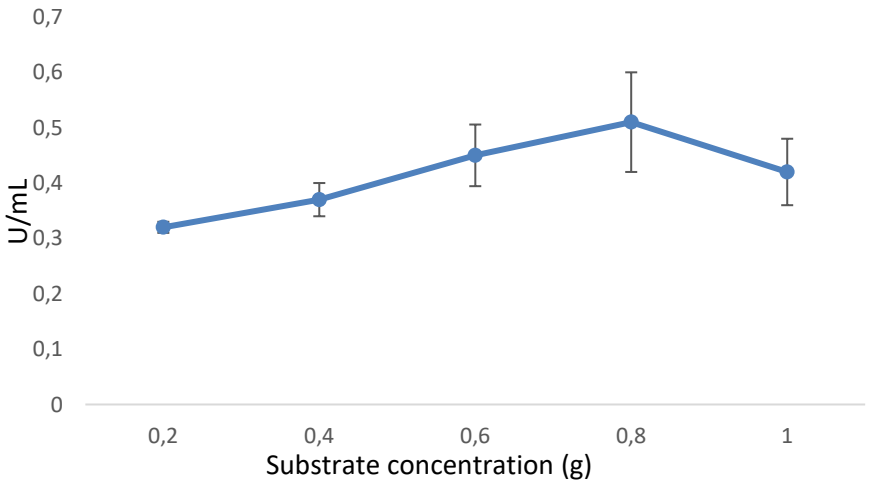

Figure 6. Optimization of substrate concentration for amylase production.

3.5. Characterization of amylase

The isolated amylase was found to have optimal activity at 40 ^{0}C and pH 5 (results not shown). Most salt lakes are found to be with alkaline pH. Anupama and Jayaraman (2011) have isolated halotolerant α-amylase from *Bacillus aquimaris* VITP4 stated that optimal activity in the pH range of 7.5 – 9.5 at 40 ^{0}C [45]. Al-ZaZaee *et al.* (2011) isolated α-Amylase from Halophilic *Bacillus cereus* Ms6 has maximum activity at 45 ^{0}C and pH 7.0 [46].

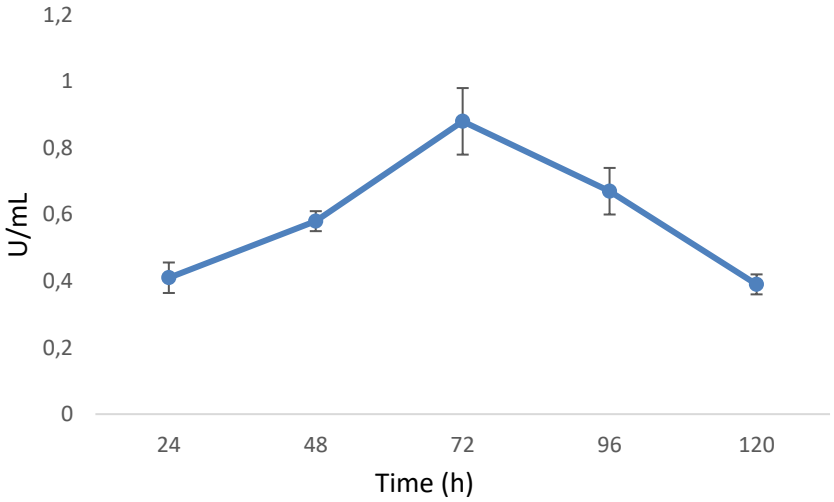

Figure 7. Optimization of time for amylase production.

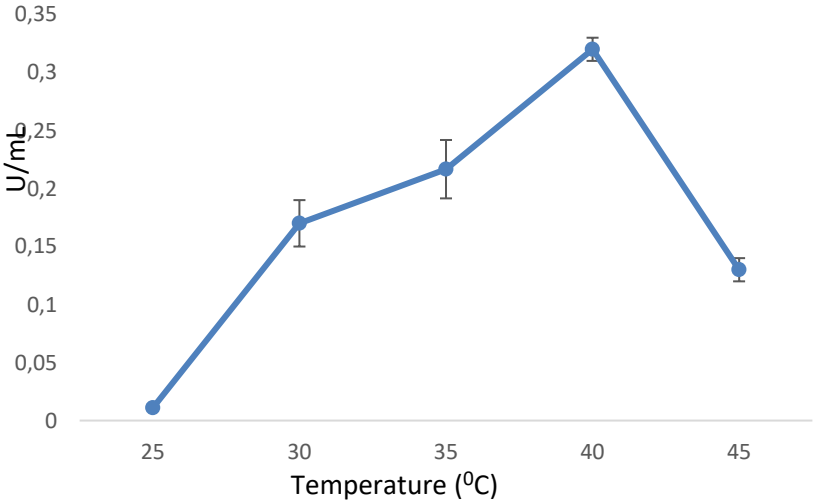

Figure 8. Optimization of temperature for amylase production.

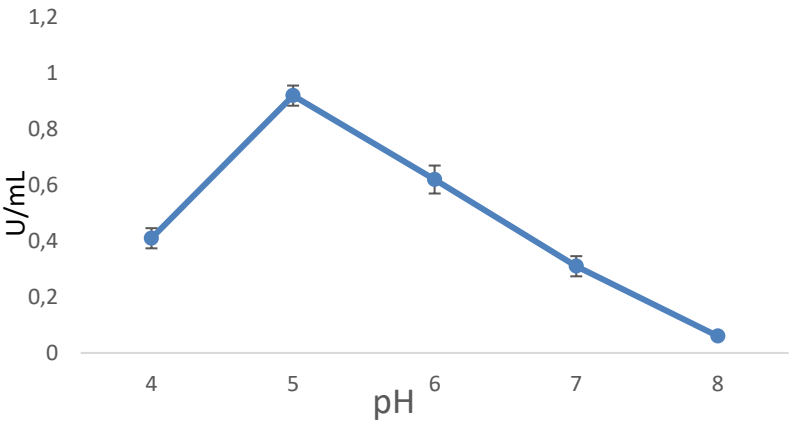

Figure 9. Optimization of pH for amylase production.

3.6. Antimicrobial susceptibility study.

Antibacterial susceptibility test for pigment was done against two pathogenic microorganisms - *Escherichia coli* and *Staphylococcus aureus*. There was no significant antibacterial potential shown by the pigment, whereas the *Streptomyces sp* MA05 extract showed activity at 100 µg concentration against *Escherichia coli.* (Table 6). Halophilic bacteria

isolated from Ratnagiri coastal area, Maharashtra, India showed antibacterial activity against Gram-negative bacteria like *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and Gram-positive bacteria - *Bacillus subtilis* [47]. Moderate halophilic *Streptoverticillium* of Bejaia [48] showed antibacterial activity against six pathogenic organisms.

Table 6. Antimicrobial activity of pigment and extract.

S.no	Source	Zone of inhibition (mm)							
		Pigment				*Streptomyces* sp MA05 extract			
		25	50	75	100	25	50	75	100
1.	*E.coli*	-	-	-	-	-	-	-	+
2.	*S.aureus*	-	-	-	-	-	-	-	-

- - no activity, + - showed activity

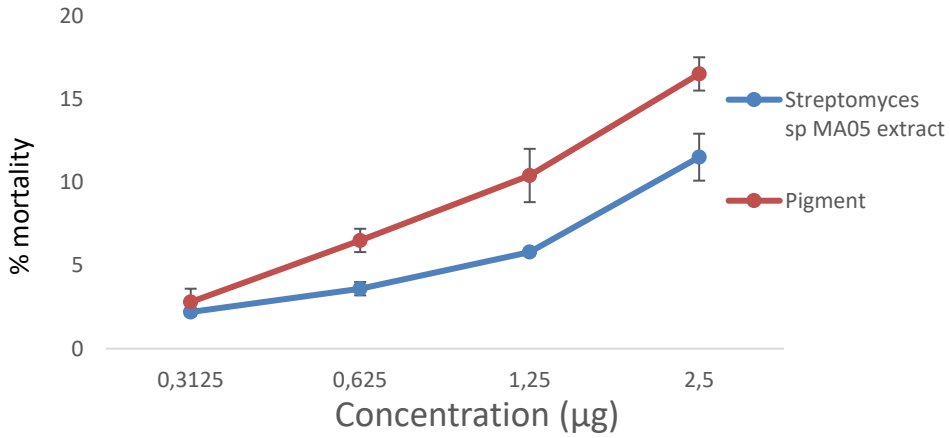

Figure 10. Cytotoxicity of *Streptomyces* sp MA05 extract and pigment against VERO cell lines.

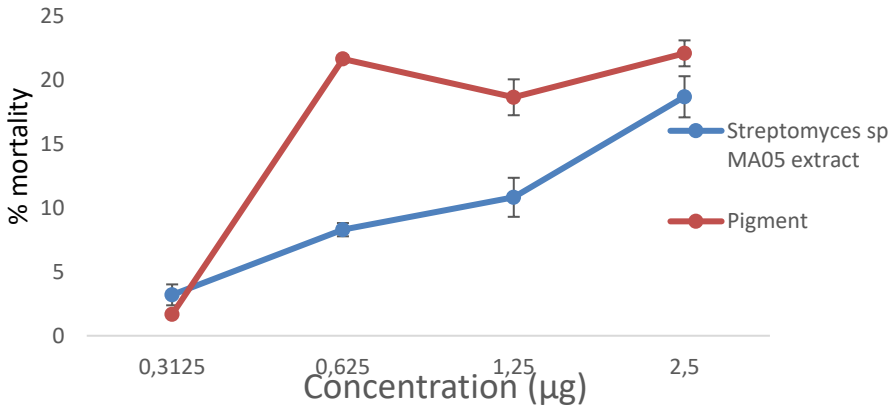

Figure 11. Cytotoxicity of *Streptomyces* sp MA05 extract and pigment against Hep G2 cell lines.

3.7. Anticancer activity.

There was no significant activity by pigment and *Streptomyces sp* MA05 extract against Hep G2 and Vero cell lines. (Fig 10 and 11). However, Abbes *et al.* (2013), who worked on pigment from *Halobacterium halobium* isolated from a Tunisian solar saltern, where the bacterial carotenoid extracts have inhibited Hep-G2 [49]. Sudha and Masilamani (2012) isolated bioactive metabolite producing *Streptomyces avidinii* strain SU4, which inhibited the proliferation of HepG2 cells [50]. In our study, both did not have the constituent, which has anticancer activity.

4. Conclusions

Two organisms were chosen for this study, which was isolated from the salt lake of Chennai. One was orange pigmented – *Halobacterium* sp MA06, and the other was chalky white colony-forming – *Streptomyces* sp MA05. The growth condition for both the organisms was optimized. Pigment production was optimized, and the pigment was found not to have antibacterial and anticancer activity. The chloroform extract of *Streptomyces* sp MA05 was found to have antibacterial activity and also to produce amylase. Amylase production was optimized, and the amylase was characterized.

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Conflicts of Interest

The authors declare no conflict of interest.

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