Production, substrate specificity and immobilization studies of agarase enzyme produced by marine bacterial isolates

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ABSTRACT

Agarase enzyme mainly obtained from marine bacteria, classified as alpha agarase and beta agarase. Production of enzyme was carried out from isolates obtained from marine water as well as marine animals (shell and shrimp) samples using MMS medium. Two isolates SE1 and SE4 gave maximum zone (21mm and 22mm respectively) of hydrolysis out of 8 isolates. Substrate specificity was performed using three different substrate Agar, Agarose, Starch and was found highest with starch as substrate by SE1 isolate as well as by SE4 isolate. Mutagenesis was carried out by both Physical and chemical methods; isolate SE1 and SE4 gave best results with starch as substrate. In immobilization studies SE4 isolate; crude enzyme gave best activity with agarose as substrate whereas stored immobilized crude enzyme gave best results with starch as substrate. While studying different optimization conditions it was found that SE1 isolate gave maximum activity at pH 5 and at temperature 25°C and 30°C with agar and starch as substrate respectively and SE4 isolate gave maximum activity at pH 6 and pH 8 with substarte agarase and at temperature 25°C with agar as substrate. By analyzing SDS-PAGE, molecular weight of crude enzyme obtained from SE1 and SE4 were estimated to be 200kDa and between 44.3 to 66.4kDa respectively. So, it may be possible that SE1 isolate produced agarase enzyme while SE4 isolate produce amylase as well. It can be said that isolates are able to produce both agarase and amylase enzyme at same time with different substrates.

Key words– Agarase, MMS medium, Mutagenesis, Immobilization, Substrate Specificity

1.INTRODUCTION

Agar, which can be degraded by agarase, is composed of agarose and agaropectin. Based on the mode of action on agarose, agarases are classified into two groups (alpha-agarase and beta-agarase) which hydrolyze a-1, 3 linkages and b-1, 4 linkages in agarose, respectively. Agarases are mainly obtained from marine bacteria. To date, a number of micro-organisms have been reported to secrete agarase, mainly in a marine environment, either in the sea water, in marine sediments or associated to red algae (Lavilla- Pitogo 1992; Schroeder et al. 2003). Those agarolytic bacteria belong to various genera including *Vibrio* (Macianet al. 2001; Zhang and Sun 2007), *Pseudomonas*,*Alteromonas* (Wang et al. 2006), *Microbulbifer* (Ohta et al. 2004), *Thalassomonas* (Jean et al. 2006), *Salegentibacter* (Nedashkovskaya et al. 2006), *Zobellia* (Jam et al. 2005), *Agarivorans* (Kurahashi and Yokota 2004) and *Pseudoalteromonas* (Chiura and Tsukamoto, 2000; Ma et al. 2007). There are also agarase-producing bacteria from nonmarine environments, like the ones from Lowland River (Agbo and Moss 1979), soil (Stanier 1942; Suzuki et al. 2003) and plant root (Hosoda et al. 2003).

Agarase producing bacteria has been isolated from different environments. Most of the agarase producing isolates were found to be of marine origin. However, Feng et al. (2012) isolated an agarolytic *Rhodococcus sp*. from printing and dyeing wastewater. Agarolytic bacteria have also been isolated from terrestrial soil (Suzuki et al., 2003; Hosoda et al., 2006; Lakshmikanth et al., 2006b). In recent years, several novel agarase producing strains were isolated from marine environments. Agarolytic *Aliagarivorans marinus* and *Aliagarivorans taiwanensis* (Jean et al., 2009), *Simiduia agarivorans* (Shieh et al., 2008) were isolated from seawater. *Flammeovirga sp.* MY04 (Han et al., 2012), *Agarivorans sp*. isolated from marine sediments were found to pro-duce β–agarase. Seaweeds were found to be one of the primary sources of agarolytic bacteria in the marine environment (Lee et al., 2013; Oh et al., 2010). Agarolytic bacteria have also been isolated from the gut of mollusks (Fu et al., 2008; Jung et al., 2012).

Applications of agarases are well-known in the areas of food, pharmaceuticals, cosmeceuticals, and biotechnology. The neoa-garooligosaccharides have been considered to have high economic value, because of their physiological and biological activity with-out toxicity, as GRAS. These oligosaccharides especially neoagar-otetraose and neoagarohexaose exhibit antioxidative activity, scavenging hydroxyl free radicals and superoxide anion radicals and inhibiting lipid peroxidation (Wang et al., 2004; Wu et al., 2005). Additionally, neoagarooligosaccharides acts as a low-calorie.

In recent study, production of agarase producing microorganism and the effect of different conditions on the production of agarases have been described.

2. MATERIALS AND METHODS

2.1 Sampling, isolation, purification of agarolytic marine bacteria

Marine samples such as seawater, shells, and shrimps were collected from Tithal beach and Hingraj beach in Valsad on the coast of Arabian Sea in the state of Gujarat, Devka beach of capital city of the Indian union territory of Dadra and Nagar haveli and Daman and Diu. 2ml of Sample suspended in the sterilized minimal mineral salts medium (MMS) (Lakshmikanthetal. 2006b) containing (g/L) K_2HPO_4 (1.2), KH_2PO_4 (0.3), $MgSO_4$ (0.1), $FeCl_3$ (0.1), NH_4NO_3 (1.0), $CaCl_2$ (0.1) and agar (1.0) (pH 7.0). After incubation on rotary shaker at 170 rpm at 37 °C for 72h, a loop full of the medium was streaked on MMS media plates containing 2.5% agar. Following incubation, the colonies exhibiting an obvious clear hydrolysis zone around the colony or pit formation, indicative of agar degradation were selected as agarolytic bacteria and purified by repeated streaking.

2.2 Secondary screening of agarolytic marine bacteria:

2◦ screening of agarolytic activity was carried out by spot inoculation of purified culture on MS agar plates made with seawater and over laying with Lugol's iodine after incubation for 72 h at 37 °C . The clear zone around the colony indicates the agarolytic activity. Isolates giving maximum zone was selected for further studies. (T.Leema Roseline, N.M Sachindra., 2016)

2.3 Agarase Activity Measurements:

Screened cultures (SE1 and SE4) were inoculated to the Artificial Sea Water broth containing (g/L) Tris HCL (6.1$MgSO_4$ (12.3), KCL (0.74), $(NH_4)_3HPO_4$ (0.31), Yeast extract (0.5) $CaCl_2$, agar (0.5). The pH was adjusted to7.2 before sterilization at 121 °C for 15min. The culture was incubated at 37 °C on the orbital shaker at 180rpm. After 72h of incubation at room temperature, the production media was centrifuged at 8000 rpm for 15min at 4 °C and the supernatant collected was taken as a crude extracellular enzyme.

Agarase activity was measured by the release of reducing sugars according to the 3, 5-dinitrosalicylicacid (DNS) method (Miller, 1959). Briefly, 1ml of the crude enzyme solution was mixed with 1ml of substrate (agar agarase and starch) vortexed and incubated for 30min at 37 °C . After incubation, 1ml of DNS solution was added to the mixture and heated in boiling water bath for 10mins, cooled, and absorbance was measured at 540nm.

2.4 Substrate specificity:

Enzyme activity was also performed using different substrates like 0.1% starch, 0.1% agarose and 0.1% cellulose. Enzyme activity was carried out as mentioned above.

2.5 Mutagenesis of agarolytic marine bacteria:

Mutagenesis was carried out by both physical and chemical method. In physical mutagenesis 1ml of the culture of both SE1 and SE4 was collected in the sterile empty petri dishes and UV exposure was given for 10s, 20s, 30s, and 40s respectively. Exposed content was then inoculated in 50ml of sterile ASW broth and incubated for 72h at room temperature. After incubation enzyme activity was performed.

Chemical mutagenesis includes two mutagens: Acridine orange (100µg/ml) and Ethidium bromide (50µ/ml). Bacterial cultures were inoculated with respective chemical and incubated for 4 hour and agarolytic activity was carried out with DNS method for both SE1 and SE4 from crude enzyme.

2.6 Immobilization Studies:

2.6.1 Immobilization of non-mutant & mutant agarolytic bacteria:

Immobilization was carried out by entrapment method in which enzyme is trapped in insoluble beads of sodium alginate. In a syringe 9ml of 3% sodium alginate and 1ml of crude enzyme was taken and beads were formed by dropping the mixture into 50 ml chilled 1M calcium chloride solution. After 120 min of curing, beads were recovered by filtration following washing with phosphate buffer and then dried on filter paper. Enzyme activity was then performed with DNS method for both mutant and non-mutant bacteria.

2.6.2 Storage time analysis:

The immobilized beads of crude enzyme, physically mutated enzyme and chemically mutated enzyme were stored at 4°C for 24 hours and then the enzyme activity was performed with DNS method, to check the effect of storage time on agarolytic activity.

2.7 Effect of culture condition on Agarase production by selected isolates:

The effect of pH (5, 6, 7, 8, 9 and 10) and temperature (25, 30, 37, 50 and 60◦C) was evaluated by culturing the selected organism under specific condition, by varying one parameter and keeping the other condition constant. Enzyme activity was then measured by DNS method.

2.8 Analysis of SDS-PAGE for extracted crude enzyme:

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel with, Polyacrylamide separating gel (12%) and Stacking gel (4%). Samples were treated with loading dye and directly loaded on the Gel. 40 µl sample volume was loaded on the gel. The gel was stained with Coomassie brilliant blue R-250 and destained. The Broad range molecular weight marker was used for the molecular weight determination.

3. RESULTS AND DISCUSSION

3.1 Sampling, isolation, purification of agarolytic marine bacteria

From seawater, shrimp sample and seashell sample 3, 1 and 4 isolates were isolated respectively, which were detected agarase producer by flooding plates with iodine. Isolates were purified and preserved for further studies. All isolates were gram positive and rods or cocci morphology. (T. Leema Roseline, N.M Sachindra., 2016).

3.2 Secondary screening of agarolytic marine bacteria:

Spot inoculation showed that two isolates (SE1 and SE4) gave good agarolytic activity; demonstrated by zones of hydrolysis around the colony after flooding plates with iodine, and were selected for further studies. (Fig. 1). SE1 and SE4 gave 21mm and 22 mm diameter hydrolysis zone respectively. (T.Leema Roseline, N.M Sachindra., 2016)

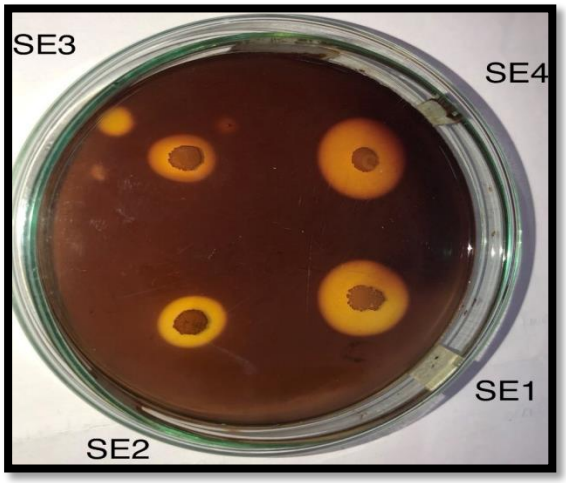


Fig. 1 Secondary Screening of Agarolytic Marine Bacterial Isolates

3.3Agarase Activity Measurement:

Agarolytic activity for both the isolates were performed by DNS method. In Agarase activity for crude enzyme, performed with Agar as substrates, **SE1 gave 27.7 µg/min/ml, while SE4 gave 025.9 µg/min/ml** (Fig. 2).

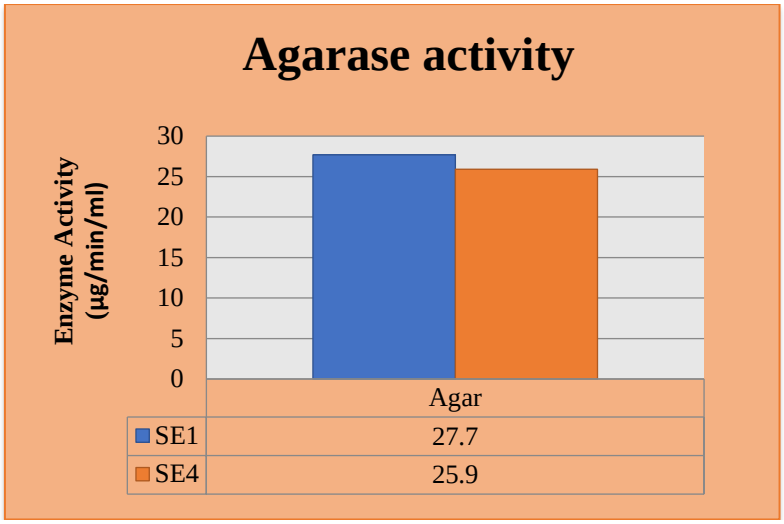


Fig. 2 Agarase Activity Given by Isolate SE1 and SE4

3.4 Substrate specificity using different substrates:

By using Agar, Agarose, Starch and Cellulose as different substrates, substrate specificity of Agarase enzyme towards these substrates was carried out. **It was found that highest activity was given by substrate starch by isolate SE1 (042.02 µg/min/ml) and by isolate SE4 (44.8 µg/min/ml), while lowest activity was given by Cellulose with isolate SE1 (9.6 µg/min/ml) and no activity was given by isolate SE4.** (Fig. 3).

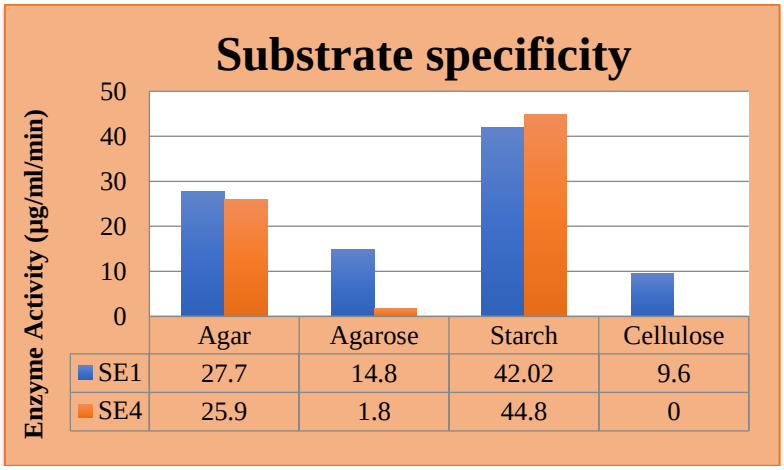
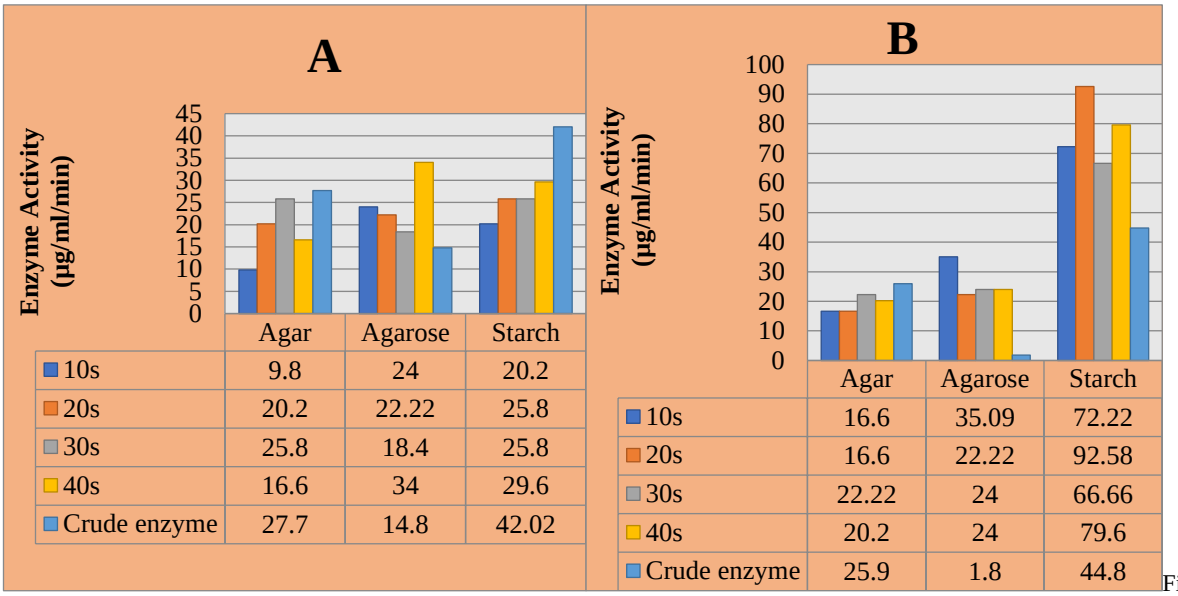


Fig. 3 Results of Substrate Specificity

3.5 Mutagenesis of agarolytic marine bacteria:

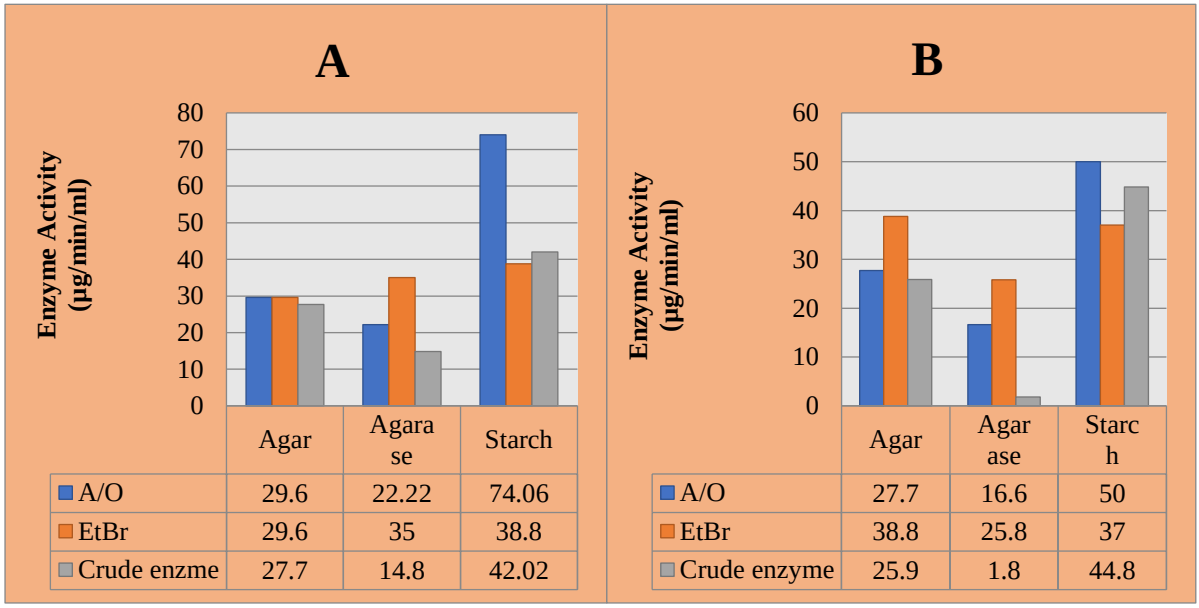
As cellulose gave nil activity it was not used in further studies. Activity was performed using three different substrates (Starch, Agarose and agar). Physical and Chemical both type of mutagenesis techniques was used for both the isolates**. In Enzyme activity for crude enzyme, SE4 isolate with substrate Starch gave highest activity (44.8 µg/min/ml).** While in the study of T. Leema Roseline and N.M Sachindra in June 2016, agar gave highest activity their respective isolate**. Physical mutagenesis** was carried out for 10seconds to 40seconds for both isolates. **Best result was observed at 20seconds; isolate SE1 (29.6 µg/min/ml) gave optimum activity with starch as a substrate** (Fig. 4A) **and at 40seconds; isolate SE4 (92.58 µg/min/ml) gave optimum activity with starch as substrate** (Fig. 4B)**. Chemical mutagenesis** was performed with two dyes Acridine orange and ethidium bromide for both the isolates**. Highest result was observed with acridine orange using starch as substrate by isolate SE1 (74.06 µg/min/ml)** (Fig. 5A) **and with acridine orange using starch as substrate by isolate SE4 (50 µg/min/ml)** (Fig. 5B) **Possibly the enzyme may be amylase not agarose but due to its broad specificity it is also able to digest agar and agarose or there may be simultaneous production of agarase and amylase and both are present in crude enzyme supernatant.**



	Agar	Agarose	Starch
10s	9.8	24	20.2
20s	20.2	22.22	25.8
30s	25.8	18.4	25.8
40s	16.6	34	29.6
Crude enzyme	27.7	14.8	42.02

	Agar	Agarose	Starch
10s	16.6	35.09	72.22
20s	16.6	22.22	92.58
30s	22.22	24	66.66
40s	20.2	24	79.6
Crude enzyme	25.9	1.8	44.8

Fig. 4 Results of Effect of Physical Mutagenesis on isolates A) SE1 and B) SE4



	Agar	Agarase	Starch
A/O	29.6	22.22	74.06
EtBr	29.6	35	38.8
Crude enzme	27.7	14.8	42.02

	Agar	Agarase	Starch
A/O	27.7	16.6	50
EtBr	38.8	25.8	37
Crude enzyme	25.9	1.8	44.8

Fig. 5 Results of Effect of Chemical Mutagenesis on isolates A) SE1 and B) SE4

3.6 Immobilization studies:

3.6.1 Immobilization of non-mutant & mutant agarolytic bacteria:

Immobilization was carried out by enzyme entrapment method. In this study previously studied crude, physical and chemical mutagenesis treated enzymes were immobilized using sodium alginate and beads were used for determination of enzyme activity. **For crude enzyme immobilization best result was found for substrate agarose for isolate SE4 (127.6 µg/min/ml)** (Fig. 6).**For physical mutant immobilization best result was found at 40seconds with starch as substrate by isolate SE4 (124 µg/min/ml)** (Fig. 7). **For chemical mutant immobilization best result was found for EtBr dye using agar as substrate by isolate SE4 (92.4 µg/min/ml)** (Fig. 8). **So, crude enzyme immobilization shows maximum activity. There for it can be said that no effect on enzyme activity was observed after physical or chemical mutation by immobilizing the enzyme.**

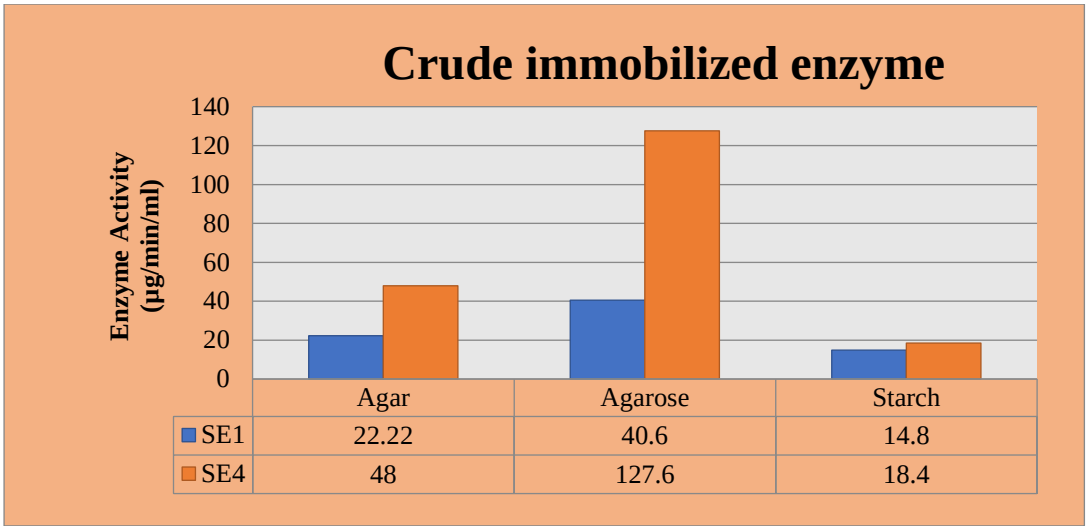


Fig. 6 Result given by crude immobilized enzyme

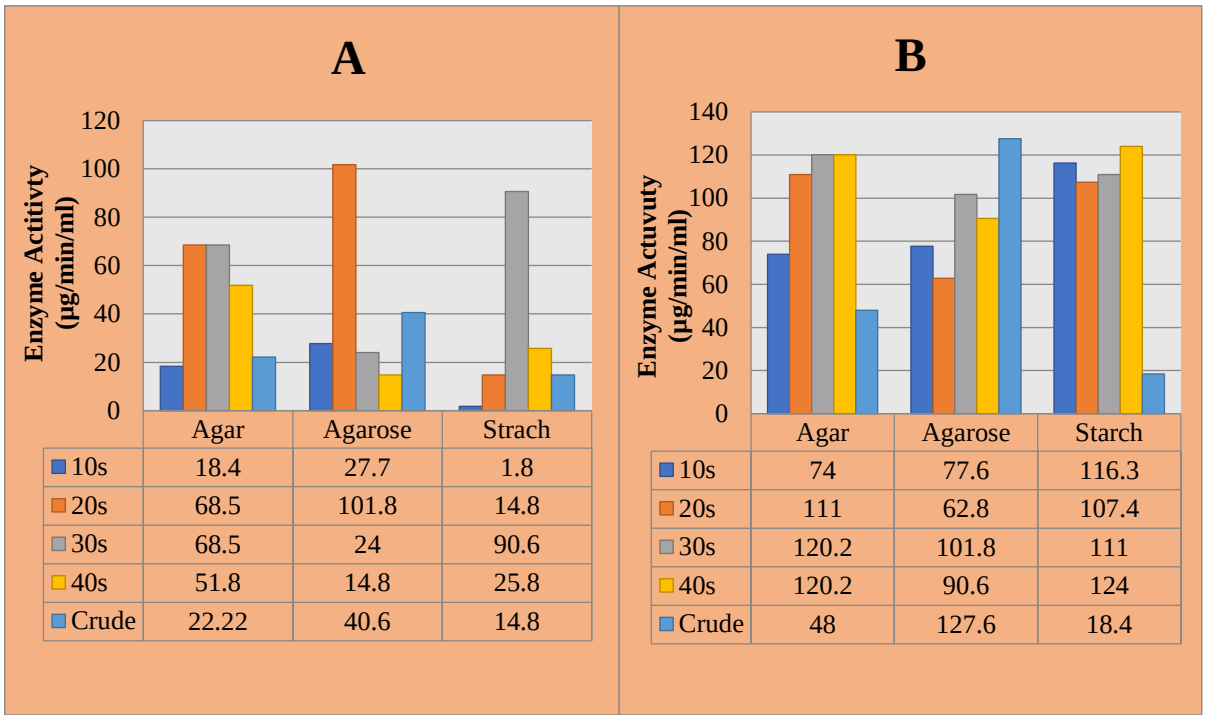


Fig. 7 Results given by immobilized enzymes Obtained after Physical mutagenesis A) SE1 and B) SE4

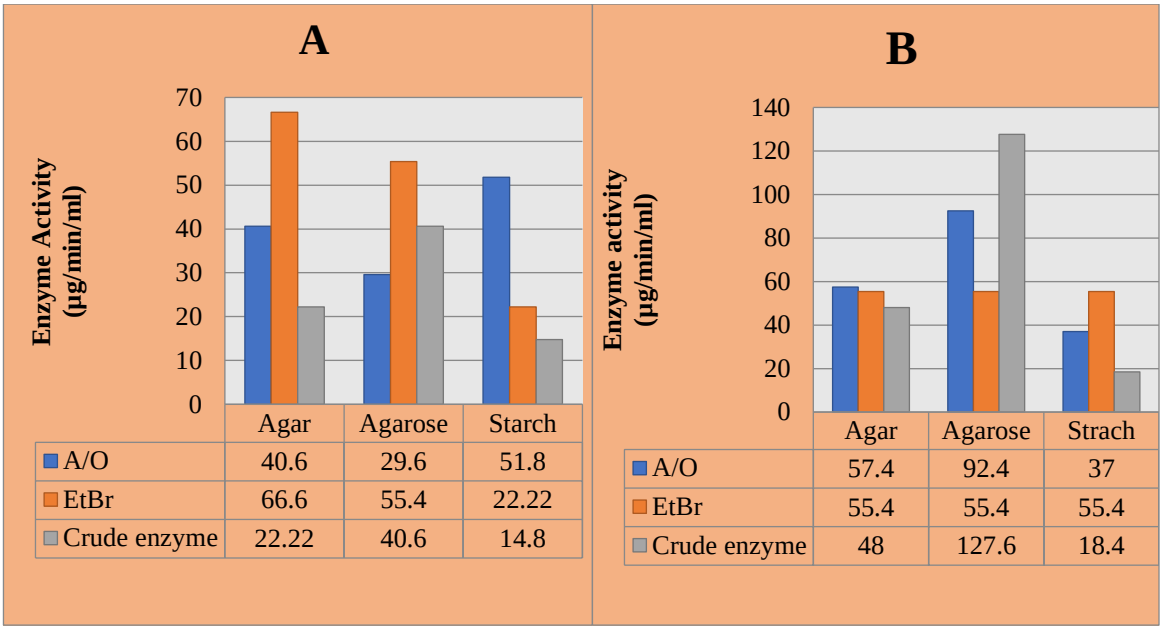


Fig. 8 Results given by immobilized enzymes Obtained after Chemical mutagenesis A) SE1 and B) SE4

3.6.2 Storage time analysis:

Storage time analysis was performed by storing immobilized enzymes (Crude immobilized enzyme, physically mutated immobilized enzyme and chemically mutated immobilized enzyme) at 4°C for 24hours and the activity of each was then compared with respective immobilized enzyme which was stored at 0 hour. Enzyme activity was performed using three substrates (Starch, Agarose and Starch). **By storing crude immobilized enzyme, activity was found highest for substrate agarose was given by isolate SE1 (133.2 µg/min/ml) after 24 hours (Fig. 9) For immobilized physical mutant result was highest at 40s using substrate starch by isolate SE4 at 0 hour (124 µg/min/ml) (Fig. 10). While immobilized chemical mutant shows highest activity using EtBr dye with substrate agar by isolate SE1 at 0 hour (92.4µg/min/ml) (Fig. 11). So, from above observations it can be said that highest activity was given when immobilized crude enzyme was stored at 4°C for 24hours.**

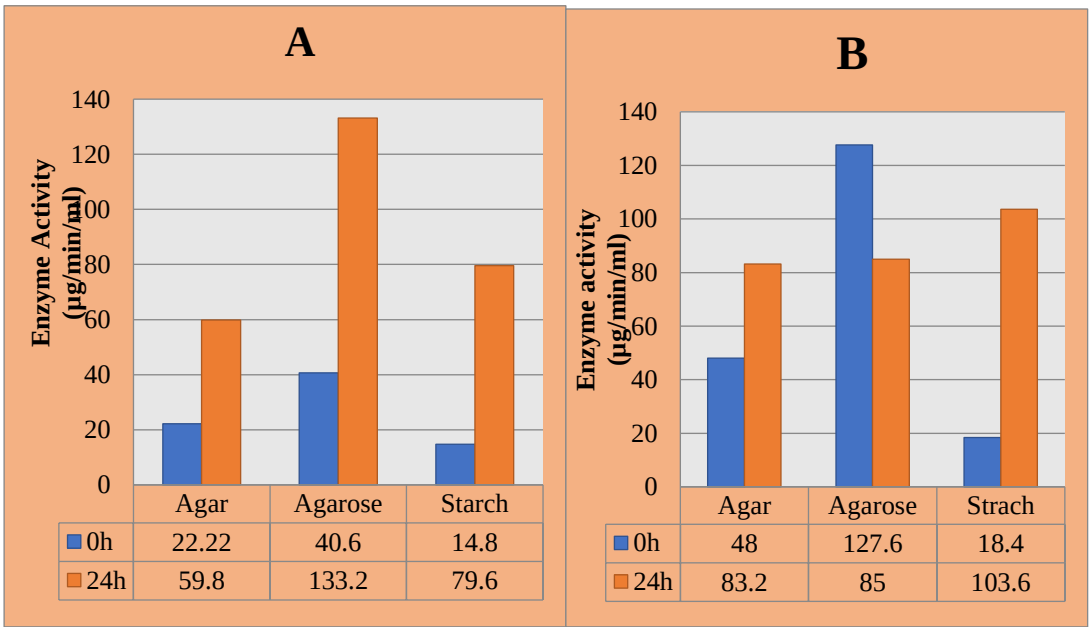


Fig. 9 Result of Effect of Storage Time Analysis of Crude Enzyme

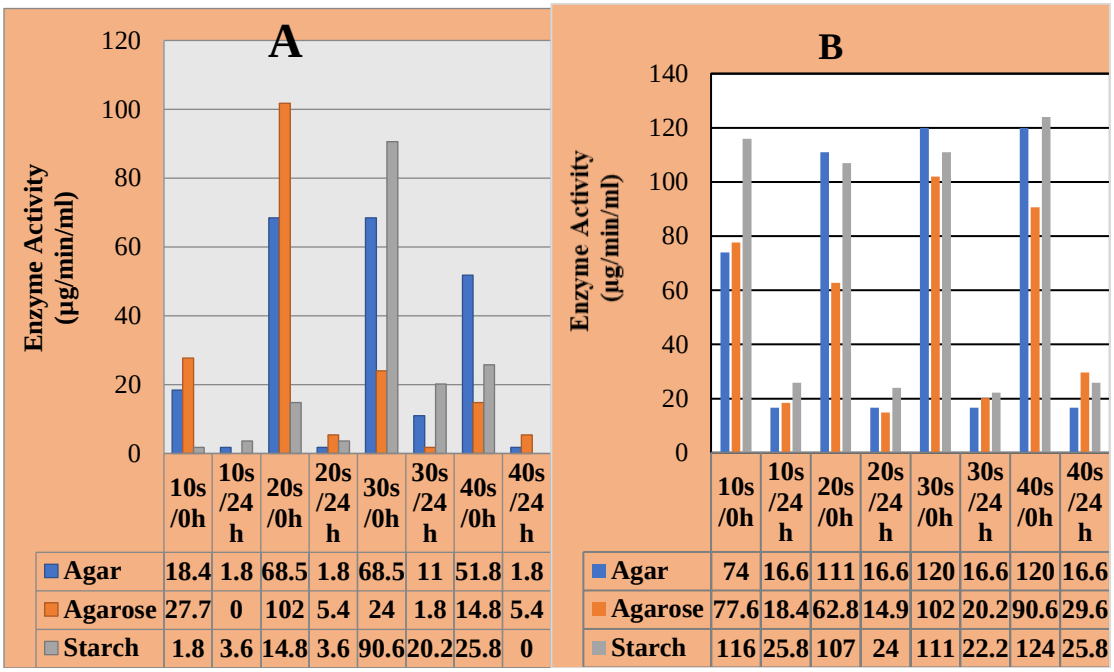


Fig. 10 Result of Effect of Storage Time Analysis of UV mutated enzyme Given by isolate A) SE1 and B) SE4.

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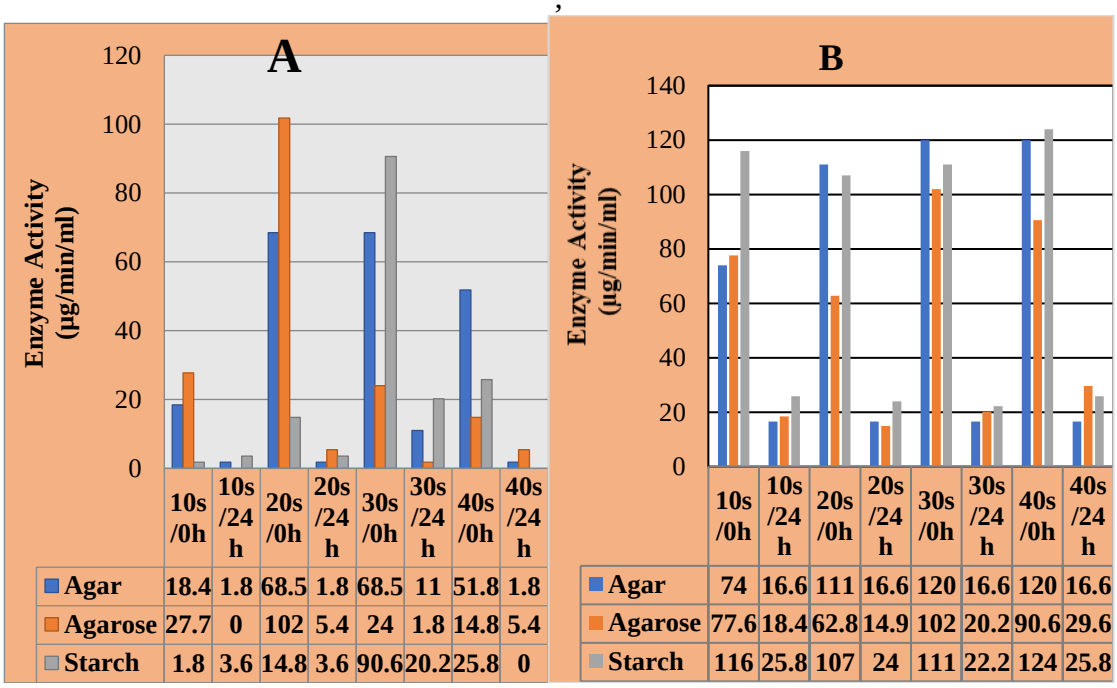
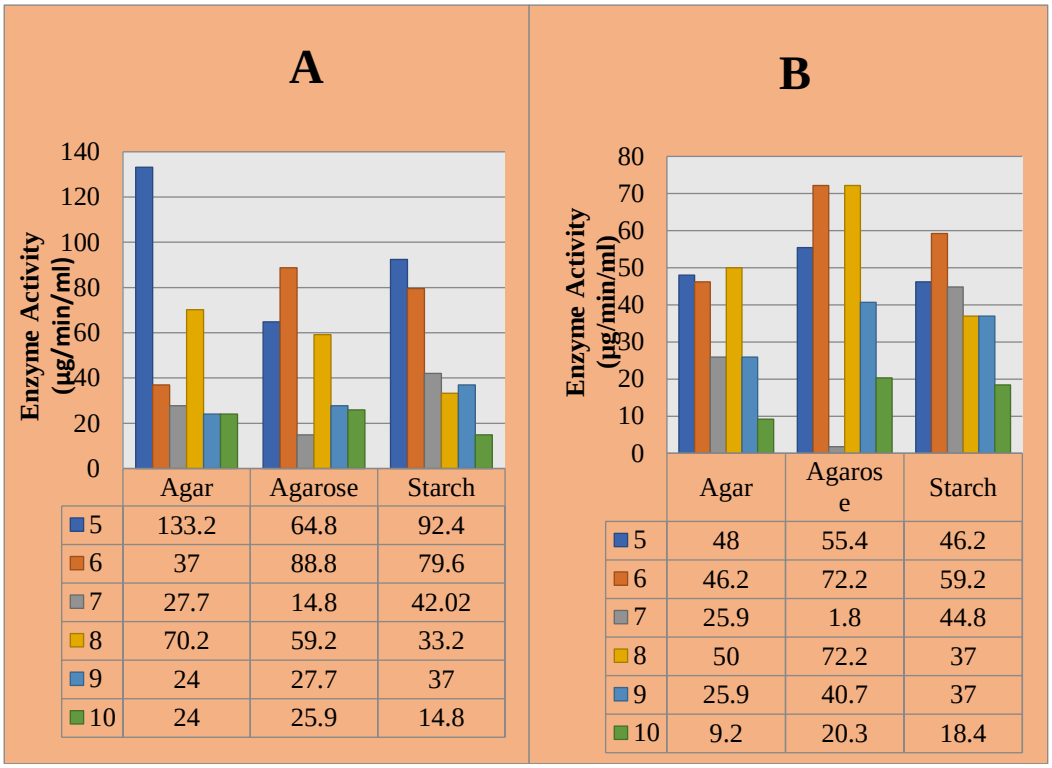


Fig. 11 Result of Effect of Storage Time Analysis of Chemically mutated enzyme Given by isolate A) SE1 and B) SE4.

3.8 Effect of culture condition on Agarase production by selected isolates:

Optimum pH for both the isolate SE1 and SE4 was carried out by varying the pH but keeping all other condition same. Different pH (5-9) was performed for enzyme activity using different substrates (Agar, Agarose and starch). As shown in (Fig. 12) it can be said that for **isolate SE1, pH5 with substrate agar, pH6 with substrate agarose and pH5 with substrate starch gave best results. For isolate SE4, pH8 with substrate agar, pH6 and pH8 with substrate agarose and pH6 with substrate starch gave best results.** For temperature optimization activity with different substrates (Agar, Agarose and Starch) was carried out with various temperature (25°, 30°, 37°, 50°and 60°C). From fig. it can be said that for **isolate SE1, 37°C with substrate agar, 25°C with substrate**

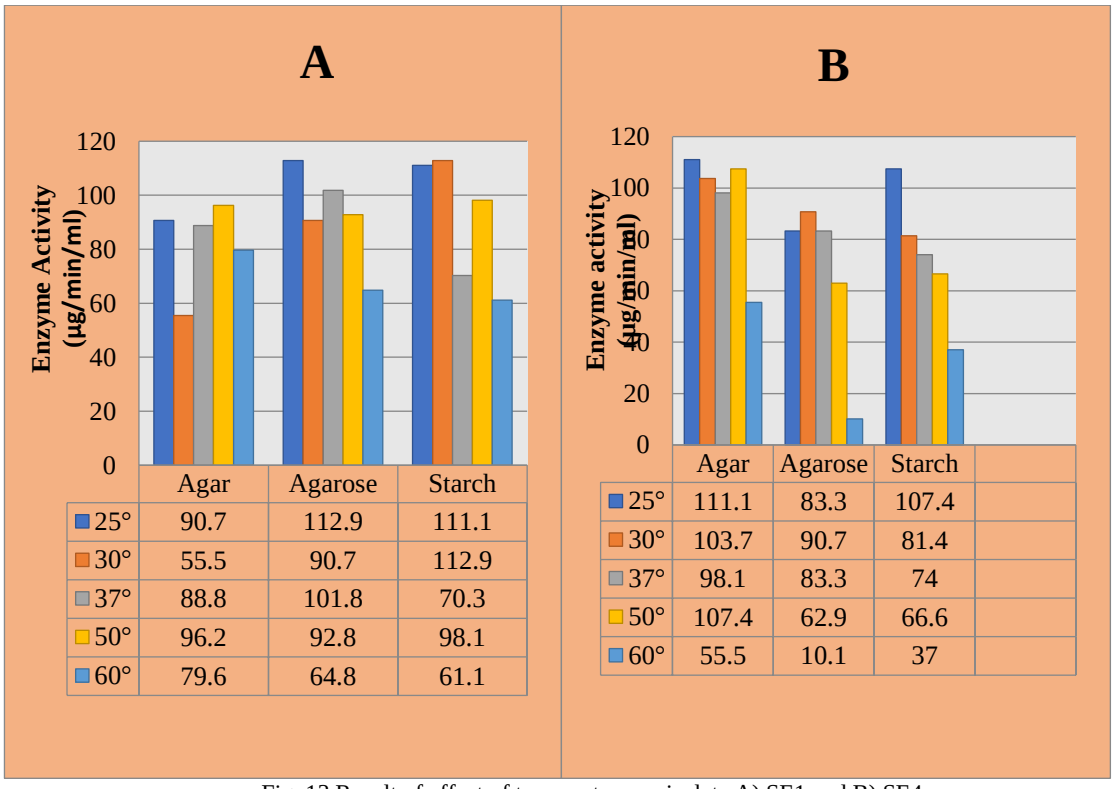
agarose and 30°C with substrate starch gave best results. For isolate SE4, 25°C with substrate agar, 30°C with substrate agarose and 25°C with substrate starch gave best results (Fig. 13).



	Agar	Agarose	Starch
5	133.2	64.8	92.4
6	37	88.8	79.6
7	27.7	14.8	42.02
8	70.2	59.2	33.2
9	24	27.7	37
10	24	25.9	14.8

	Agar	Agarose	Starch
5	48	55.4	46.2
6	46.2	72.2	59.2
7	25.9	1.8	44.8
8	50	72.2	37
9	25.9	40.7	37
10	9.2	20.3	18.4

Fig. 12 Result of effect of pH on isolate A) SE1 and B) SE4.



	Agar	Agarose	Starch
25°	90.7	112.9	111.1
30°	55.5	90.7	112.9
37°	88.8	101.8	70.3
50°	96.2	92.8	98.1
60°	79.6	64.8	61.1

	Agar	Agarose	Starch
25°	111.1	83.3	107.4
30°	103.7	90.7	81.4
37°	98.1	83.3	74
50°	107.4	62.9	66.6
60°	55.5	10.1	37

Fig. 13 Result of effect of temperature on isolate A) SE1 and B) SE4.

5.9 Analysis of extracted crude enzyme SDS-PAGE:

Molecular weights of agarase are highly divergent ranging from 20 to 360 kDa. (Xiou Ting Fu and Sang Moo kim,2010). As per one study the enzyme is a dimer with 360 kDa, so it's one denatured chain contain 180 kDa molecular weight (Xiou Ting Fu and Sang Moo kim,2010). SDS-PAGE was done from extracted supernatant of both isolate to verify the presence of 180 kDa molecular weight protein as it may contain agarase enzyme**. For SE1 isolate band was observed near 200 kDa marker. Moreover SE 4 also gave good amylase activity and molecular weight of amylase is around 54 k Da** (Fig. 14). So, it may be possible that SE4 isolate produce amylase enzyme as well. As it can be observed in below figure **that protein concentration was comparatively less in SE 1 supernatant and so yield lighter bands, whereas SE 4 supernatant's SDS-PAGE analysis showed darker and somewhat clearer bands. Bands present near to 200kDa marker protein are suggestive of possible presence of agarase enzyme whereas presence of bands between 44.3 to 66.4 kDa marker proteins suggests possibility of amylase enzyme as well.**

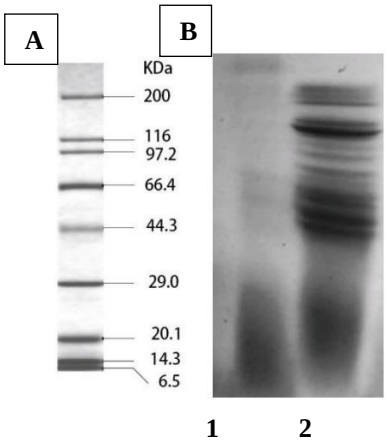


Fig. 14 Result of SDS-PAGE Analysis of extracted crude enzyme. A) Protein mass markers
B) Lane 1 showing SDS-PAGE of isolate SE1 and Lane 2 showing SDS-PAGE of isolate SE4.

4. CONCLUSION

Marine ecosystem is an enormous source of various microbes capable to produce potentially important products. Agarolytic bacteria are one of them, as marine algae contain good amount of polysaccharide agar. In this study two highest agarase activity yielding isolates SE1 and SE4 were obtained from a marine shrimp. During substrate specificity study SE4 isolate gave maximum activity with starch as substrate. After performing 20 seconds UV mutagenesis SE4 isolate gave maximum activity with substrate starch. Crude enzyme of SE4 isolate gave maximum activity with substrate agarose after immobilization. At 4°C SE1 isolate gave maximum activity with substrate agarose. At pH 5 SE1 isolate gave maximum activity with substrate agar and at pH 6 and 8 isolate SE4 gave maximum activity with substrate agarase. At 25°C and 30°C temperature SE1 isolate gave maximum activity with substrate starch and at 25°C temperature SE4 isolate gave maximum activity with substrate agar. After running SDS-PAGE SE1 isolate gave clear band at 200 k Da suggesting presence of agarase while SE4 gave bands between 44.3 to 66.4 k Da suggesting presence of amylase as well. Agar is primarily a polysaccharide of galactose and starch is of glucose. Glucose and galactose are epimeric aldohexose sugars. Only a small structural change here may be responsible for enzyme activity in presence of agar, agarose and starch. Possibly the enzyme may be amylase not agarose but due to its broad specificity it is also able to digest agar and agarose or there may be simultaneous production of agarase and amylase and both are present in crude enzyme supernatant.

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