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Activity optimisation of extracellular agarases produced by agarolytic bacteria *Flammeovirga yaeyamensis*AM5.A, *Aliagarivorans marinus*AM17.E1 and *Aliagarivorans taiwanensis*A69.B2, isolated from coral reef ecosystems

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ABSTRACT

The present study was undertaken to standardise the conditions for activity optimisation of agarases produced by three distinct strains of agar degrading bacteria *viz, Flammeovirga yaeyamensis*AM5.A*, Aliagarivorans marinus*AM17.E1 and *Aliagarivorans taiwanensis*A69.B2 (GenBank Acc. Nos. MT473965; MT475710 and MT473967 respectively) isolated from coral reef ecosystems along the southern coast of India. Agarase from *F. yaeyamensis* exhibited highest activity at 40°C, while *A. marinus* and *A. taiwanensis* agarases had peak activity at 50°C. The optimal pH and incubation time for agarases from all the three strains were 7.0 and 45 min respectively. The partially purified enzyme-extracts from the three strains were further studied for their responses to the presence of various metal ions (Cu^{2+}, K^{+}, Hg^{2+} Mn^{+}, Na^{+} and Ca^{2+}); a chelating agent, ethylenediamine tetraacetic acid (EDTA); a reducing agent, mercaptoethanol and a serine protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF). Enzyme characterisation results clearly indicated the sensitivity of all the three agarases to Hg^{2+} and Na^{+} ions. Cu^{2+} ions were found inhibitory to the enzyme from *A. taiwanensis* and *A. marinus*. However, *F. yaeyamensis* derived agarases remained unaffected even at 5 mM concentrations of Cu^{2+}. Presence of K^{+} ions evidently suppressed the agarases from *F. yaeyamensis* and *A. marinus*. EDTA, mercaptoethanol and PMSF were found to be inhibitors of the enzyme activity, while Mn^{+} and Ca^{2+} had additive effect. The results of the study indicated potential of the bacterial strains investigated in this study as prospective sources of agarases.

Keywords: Agar degrading bacteria, Chelating agent, Metal ions, pH, Reducing agent, Serine protease inhibitor, Temperature, Time of incubation

Introduction

Heterotrophic marine bacteria steer the ocean carbon cycle by recycling carbohydrates from algae, the base of marine food webs (Azam *et al.*, 2007). Algal polysaccharides are highly heterogeneous and are metabolised by sophisticated systems of enzymes (Davies *et al.*, 2002) which work in a concerted manner for polymer degradation. Since the major share of oceanic carbon resources are contributed by these carbohydrates, it is fundamental to study the processes and the enzymes involved in the carbohydrate cycling, especially by marine bacteria. Diverse niches of the marine ecosystems are underexplored sources of bacterial enzymes with novel catalytic and therapeutic properties. Industrial scale application necessitates a focused approach on their enzymatic properties. Among the various microbial degradative enzymes, agarases need a special mention, owing to their biotechnological, industrial and medicinal applications. Agarase is a specific glycoside hydrolase (GH) enzyme that enables the bacteria to hydrolyse and use agar as a source of energy and carbon (Pluvinage *et al.*, 2013). The agarases are classified as α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81 according to the cleavage pattern. α-agarases cleave α-L(1,3) linkages of agarose to produce agarobiose oligosaccharides with 3,6-anhydro-L-galactopyranose at the reducing end, whereas β-agarases cleave β-D-(1,4) linkages of agarose to produce neoagaro-oligosaccharides with D-galacto-pyranoside residues at the reducing end (Hassairi *et al.*, 2001; Park *et al*., 2020). Most of the agarases, purified and studied, belong to β-agarase group (Hu *et al.,* 2009; Han *et al*., 2019). Certain microorganisms like *Pseudomonas aeruginosa* AG LSL-11 (Lakshmikanth *et al.*, 2006), *Vibrio* sp. PO-303 (Araki *et al.,* 1998), possess multiple agarases. Agarolytic microorganisms belonging to diverse genera and their agarases and encoding genes have been reported (Chi *et al.*, 2012; Jung *et al*., 2017). Agarase producing bacteria are present not just in marine environments, but also in terrestrial soil (Lakshmikanth *et al.*, 2006), rivers (Agbo and Moss,

1979) and sewage (Feng *et al.*, 2012). However, in terms of novelty, marine microbial enzymes are far more appreciated (Barzkar *et al.*, 2018). Also these enzymes are considered more advantageous compared to their terrestrial counterparts, in terms of catalytic activity, sustainability and genetic manipulation (Bull *et al.*, 2000).

The present study reports investigations on agarases produced by bacterial strains isolated from coral reef ecosystems. The relatively high carbohydrate fraction in the coral organic matrix offers an attractive abode for the agarolytic bacteria, which in turn aids in host metabolism and survival (Bourne *et al*., 2016) by establishing a mutualistic association (Naggi *et al.,* 2018). In addition, the large proportion of macro algal content associated with corals can be a contributory factor to the presence of agarolytic bacteria. Occupying less than one percent of the ocean floor, coral reef ecosystem forms abode for more than 25% of known marine species and are important as storehouses of organic carbon and as regulators of atmospheric carbon dioxide, which in turn could influence climate and sea-level fluctuations (Opdyke and Walker, 1992; Vanwonterghem and Webster, 2020). However, a major portion of the coral reef microbiota is still underexplored and there exists umpteen possibilities for discovering novel bioactives with wide therapeutic and industrial importance.

Agarase enzyme finds potential application in food, pharmaceutical and cosmetic industries (Fu and Kim, 2010; Yun *et al*., 2017). Agar derived oligosaccharides are reported to possess high economic value. Some of the physiological activities of agar derived sugars include their use as skin whitening agents, anti-inflammatory agents, antioxidants and hepaprotectants (Park *et al*., 2020). Moreover, agarases can be employed for the preparation of protoplasts and extraction of labile substances from protoplasm (Jung *et al*., 2017). Agarases could also be used to recover DNA bands from agarose gel (Fu and Kim, 2010). Red seaweed derived agar is a cheaper cum healthier alternative as a thickening and gelling agent in food industry (Saha and Bhattacharya, 2010). The industrial use of agarases for enzymatic hydrolysis of agar highly demands information regarding functioning of the enzyme at different conditions. The optimum conditions *viz*. temperature, pH for enzyme reaction and time course for the completion of reaction are critical parameters that need to be understood. Also, different manufacturing conditions demand the information on the functioning of the enzyme in varying chemical environments. The performance of the enzyme in the presence of various chemical additives and its tolerance to various organic solvents is highly critical when it comes to large scale applications (Segel, 1976).

The agarolytic bacterial strains discussed in this work have already been characterised (Jayasree *et al.*, unpublished) and the present work investigated the enzymological properties of the agarases isolated from three strains *viz, Flammeovirga yaeyamensis*AM5.A, *Aliagarivorans marinus*AM17.E1 and *Aliagarovorans taiwanensis*AM69.B2, isolated from coral reef ecosystems. Agarolytic activity of *F. yaeyamensis* sp. nov. isolated from seawater was reported by Takahashi *et al*. (2006). However, data on the properties of the agarase from this species have not been published so far. Also, the class Sphingobacteria to which *Flammeovirga* belongs, is also poorly studied with respect to agarase enzyme production. Hence, to the best of our knowledge, this work offers first-hand information on agarase enzyme from *F. yaeyamensis*. Studies on agarases from *A. marinus* and *A. taiwanensis* are novel, with no data available regarding the strains or their agarases. Report by Jean *et al.* (2009) on the isolation and characterisation of *A. marinus* sp. nov. and *A. taiwanensis* sp. nov. from seawater is the only available information regarding the two strains. The present study forms the first report on agarase enzymes from bacterial strains isolated from coral reef ecosystems.

Materials and methods

Agar degrading bacterial strains and extraction of agarase enzyme

Three strains of agarolytic bacteria *viz, F. yaeyamensis* AM5.A*, A. marinus* AM17.E1 and *A. taiwanensis* A69.B2 (GenBank Acc. Nos. MT473965; MT475710 and MT473967 respectively) isolated from coral reef ecosystems were used for the study (Jayasree *et al*., unpublished). Partially purified agarase enzymes from the three strains were prepared as per Jayasree *et al*. (unpublished).

Activity optimisation of partially purified agarase enzyme by varying temperature, pH and time of incubation

In order to standardise the conditions for optimal activity of the agarase enzyme, the partially purified enzymes from all the three agar degrading bacterial strains were allowed to react with the substrate at varying temperature, pH and time periods.

Firstly, the activity of agarase enzyme was investigated at temperatures of 20, 40, 50, 60, 70 and 80°C at pH 7.2. The enzyme extracts were allowed to react with 0.2% agar substrate at these temperatures for 30 min. The effect of pH on the agarase activity was determined by altering the pH of the reaction medium to 4, 6, 7, 8 and 10. The tests were performed for 30 min at optimised temperature (which varied with strains) with 0.2% agar as the substrate. Time course of enzyme action was assessed by evaluating the enzyme-substrate reaction at different time periods *viz*,

15, 30, 45 and 60 min at the optimised temperature and pH for each strain. Residual enzyme activities after the treatments were measured by dinitrosalicylic acid (DNS) method (Miller, 1959). The amount of enzyme needed to liberate 1 µ mol of D-galactose per min from agar per mg of protein under the assay conditions was defined as one unit of agarase activity (U mg^{-1}). All tests were carried out in triplicate and the specific activities of the enzymes were calculated.

Effect of metal ions, reducing and chelating agents

Effect of various metal ions *viz*, Cu^{2+}, K^{+}, Hg^{2+} and Mn^{+} on the activity of the partially purified agarase was investigated, by supplementing different concentrations of test cations to the enzyme-substrate mixture. The enzyme extract was incubated with 0.2% agar substrate in the presence of metal salts ($CuSO_4$, KCl, $HgCl_2$ and $MnCl_2$) at two different concentrations (5 and 10 mM) and the residual enzyme activity was estimated by DNS method.

The dependence of enzyme activity on sodium and calcium ion concentrations were assayed by incubating partially purified enzyme extracts with 0.2% agar substrate at optimised temperature for 45 min at pH 7.0. Various concentrations of NaCl (100, 250, 500, 750 and 1000 mM) and $CaCl_2$ (0, 5, 10, 50, 100 mM) were tested. The residual agarase activity was estimated by DNS method.

The response of agarase to the chelating agent ethylene di amine tetra acetic acid (EDTA) and the reducing agent (ß-mercaptoethanol) were also evaluated at two different concentrations (5 and 10 mM). All the test solutions were prepared by dissolving their respective salts in phosphate buffer (pH 7.0) and the residual agarase activity was estimated by DNS method.

Effect of PMSF

Enzyme activity in the presence of freshly prepared serine protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF, dissolved in methanol) at two different concentrations (1 and 5 mM) were tested. The enzyme extract with PMSF was incubated with 0.2% agar substrate at optimised temperature for 45 min at pH 7.0 and the residual enzyme activity was estimated by DNS method.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for comparison of means among different treatments (Duncan, 1955) employing SPSS software ver. 20.0 (SPSS Inc., Chicago, Illinois, USA) and statistical significance was set at $p<0.05$.

Results

Effect of temperature, pH and time of incubation on enzyme activity

It was observed that temperature has a significant role on enzyme activity ($p<0.05$). As shown in Fig. 1a, within the range of the temperatures tested, agarase activity was found to increase with temperature and the activity reached the highest at 40-60°C (varying with strains) and subsequently decreased.

Partially purified agarase from *F. yaeyamensis* was found to be active in the temperature range of 20-80°C, with highest activity (0.90 U mg^{-1}) seen at 40°C. Further, increase in temperature resulted in reduction of activity. The enzyme exhibited lowest activity at 80°C Beyond 40°C, the dip was sharp at 50°C, after which no significant reduction in activity was observed. The steep increase in activity from 20 to 40°C signifies the critical role of temperature on the enzyme activity. Agarases from *A. marinus* and *A. taiwanensis* had the highest activity at 50°C. For both the strains, the activity was found to increase with temperature up to 50°C, beyond which the activity decreased.

pH was also found to have a significant influence ($p<0.05$) on the activity of agarase enzyme. Highest activity was recorded at pH 7.0 and lowest at pH 10.0 for agarases from all the three strains (Fig. 1b), which represents the neutral nature of the agarase enzyme. Agarase from *F. yaeyamensis* was found to have a narrow pH range for its optimal activity. Highest activity of 1.03 U mg^{-1} was observed at pH 7.0 and at pH 10.0 there was 43.6% reduction in activity. At pH 4, an activity loss of 28.15% was noted. Again at pH 6.0 (0.83 U mg^{-1}) and 8 (0.67 U mg^{-1}), 19.41 and 34.95% activity drop respectively was observed (values in parenthesis shows the residual specific agarase activity at corresponding pH levels). A higher loss was observed towards the alkaline side and these results indicate the stringent pH demand of the enzyme. Agarases derived from *A. marinus* and *A. taiwanensis* exhibited somewhat similar activity pattern at various pH levels, with highest activity seen at pH 7.0 (0.76 U mg^{-1} and 0.67 U mg^{-1} respectively). At pH 10, *A. marinus* (0.66 U mg^{-1}) and *A. taiwanensis* (0.52 U mg^{-1}) had a respective activity loss of 13.15 and 22.38%. At pH 4.0, 8.0 and 10.0, agarase from *A. taiwanensis* had the same activity of 0.52 U mg^{-1}. It is to be noted that at pH 6.0, the activity of *A. taiwanensis* agarase reduced by 15%, which implies the narrow pH demand of the enzyme. Similar results were observed for *A. marinus* agarase which showed a reduction in activity by 17% at pH 6.0 (0.63 U mg^{-1}).

As evident from Fig. 2c, the time of incubation was also found to significantly affect ($p<0.05$) the enzyme activity. Highest agarase activity was reached when the enzyme was incubated with substrate for 45 min. An increase in activity was observed up to 45 min and subsequently a decline was observed.

Effect of metal ions on enzyme activity

The effect of cations on the activity of the partially purified enzyme was investigated by supplementing the test solution with two different concentrations of cations (2 and 5 mM). Among the divalent cations tested, only Mn^{2+} (Fig. 2d) and Ca^{2+} (Fig. 2f) had a clear positive effect on the enzyme activity. In contrast, Hg^{2+} (Fig. 2c), Cu^{2+} (Fig. 2a) and K^{+} (Fig. 2b) had inhibitory effect on the enzyme activity. NaCl at 100 to 250 mM was found to be optimum for the activity of all the three agarases tested (Fig. 2e).

Agarases purified from *F. yaeyamensis* remained completely unaffected by the presence of Cu^{2+} ions, whereas $CuSO_4$ exhibited an inhibitory effect on agarases from *A. marinus* and *A. taiwanensis* (Fig. 2a). The agarase enzyme from *A. taiwanensis* showed 51.52% reduction in activity in the presence of 5 mM $CuSO_4$ (0.16 U mg^{-1}) and 27.27% reduction with 2 mM $CuSO_4$ (0.24 U mg^{-1}). *A. marinus* agarase had a respective decrease of 41.86% (0.25 U mg^{-1}) and 32.56% (0.29 U mg^{-1}) of activity with 5 and 2 mM $CuSO_4$ respectively.

KCl was also observed to inhibit activity of agarase enzyme derived from all the three bacterial strains (Fig. 2b). Only 50.51% residual activity (0.50 U mg^{-1}) was observed for agarase from *F. yaeyamensis* on treatment with 5 mM KCl *i.e.,* a loss of almost half of the activity. At 2 mM concentration, residual activity was 67.68% (0.67 U mg^{-1}). *A. marinus* agarase lost only 2.82% (0.68 U mg^{-1}) and

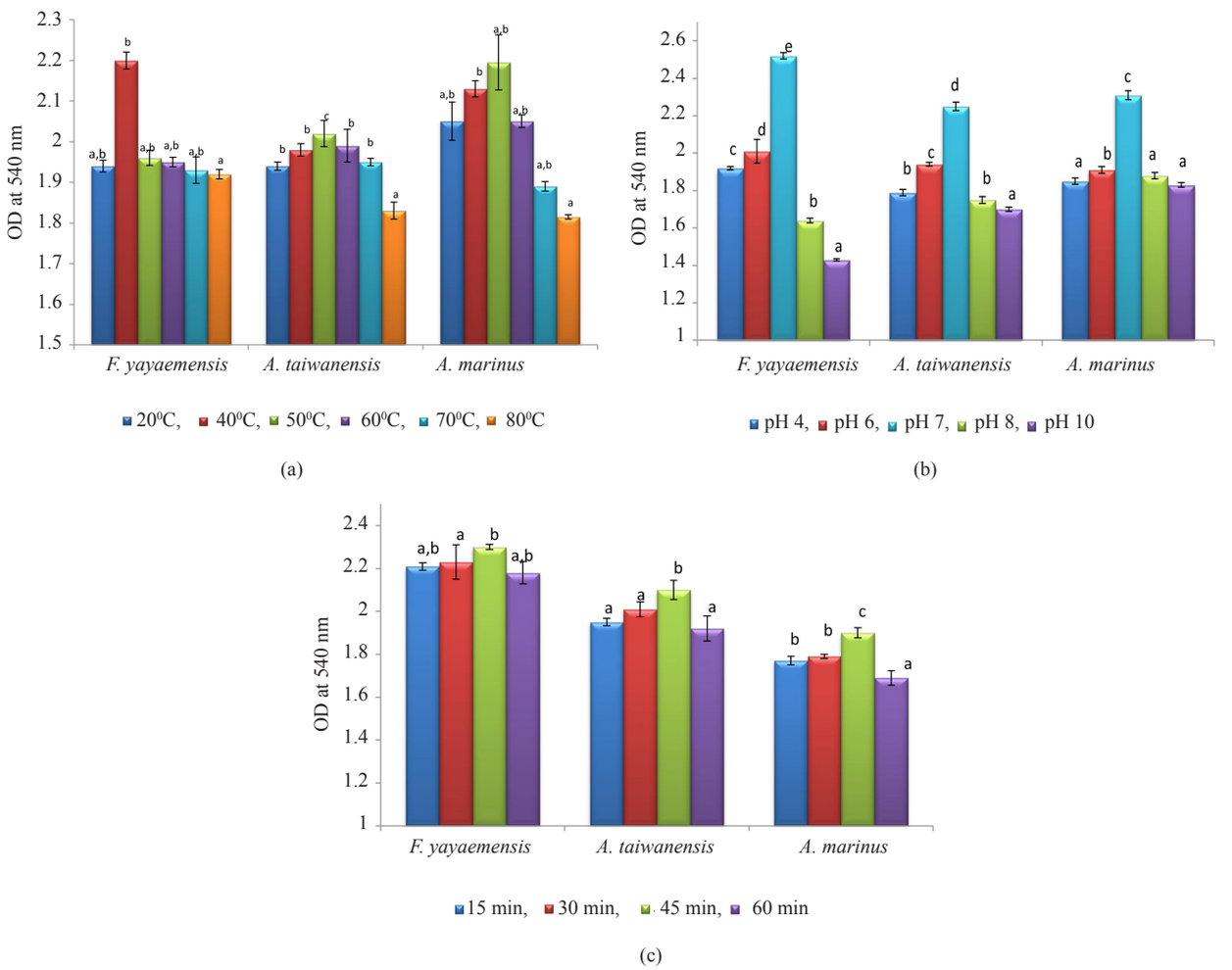

Fig. 1. Effect of (a) Temperature, (b) pH and (c) Time of incubation on the activity of partially purified agarases isolated from the three different agar degrading bacterial strains. Bars bearing different alphabets differ significantly ($p<0.05$)

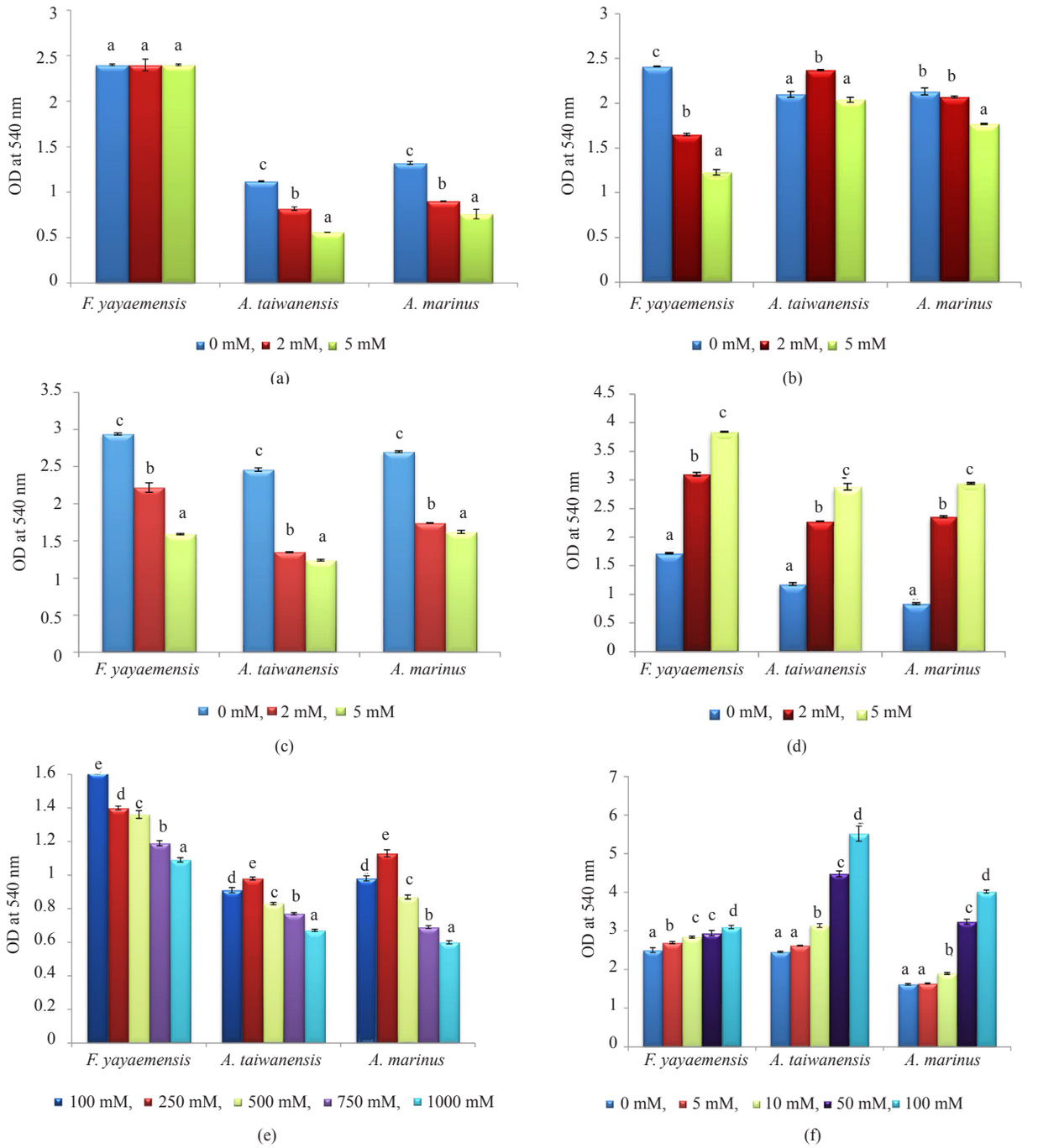

Fig. 2. Effect of (a) $CuSO_4$, (b) KCl, (c) $HgCl_2$, (d) $MnCl_2$, (e) NaCl and (f) $CaCl_2$ on activity of partially purified agarases isolated from the three agarolytic bacterial strains. Bars bearing different alphabets differ significantly ($p<0.05$)

17.14% (0.58 U mg^{-1}) of its activity respectively at 2 and 5 mM concentrations. *A. taiwanensis* agarase was also least affected by the presence of KCl with a loss of only 3.23% activity (0.60 U mg^{-1}) at 5 mM concentration, but this was preceded by a small increase in activity at 2 mM (0.70 U mg^{-1}). This denotes the slightly stimulating effect of KCl at lower concentration, which at higher concentrations exhibited an inhibitory effect. More studies on molecular level dose dependent response of agarase to KCl can substantiate this process. Among the three strains, *F. yaeyamensis* was found to be strongly inhibited by the presence of KCl.

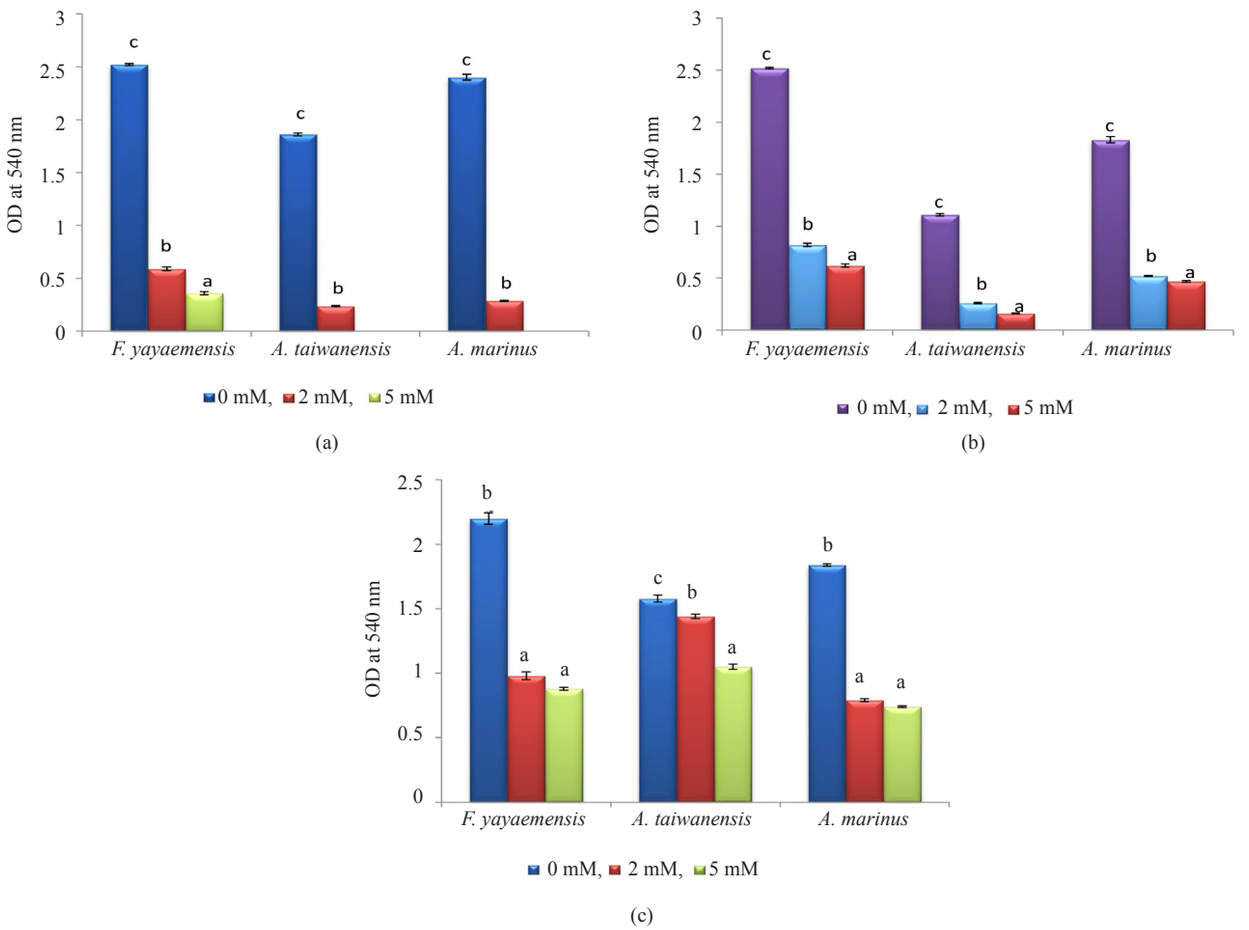

Fig. 3. Effect of (a) β-mercaptoethanol, (b) EDTA and (c) PMSF on activity of partially purified agarases isolated from agarolytic bacterial strains. Bars bearing different alphabets differ significantly ($p<0.05$)

There was a reduction of 46.72% in the activity of agarase enzyme from *F. yaeyamensis* when treated with 5 mM $HgCl_2$ (0.65 U mg^{-1}) and 25.4% reduction was recorded with 2 mM $HgCl_2$ (0.91 U mg^{-1}), which clearly indicated the inhibitory effect of $HgCl_2$ (Fig. 2c). Agarase from *A. marinus* lost 40.45% activity on treatment with 5 mM $HgCl_2$ (0.53 U mg^{-1}) and 34.83% (0.58 U mg^{-1}) with 2 mM $HgCl_2$. *A. taiwanensis* agarase lost 50.68% (0.40 U mg^{-1}) and 45.21% (0.36 U mg^{-1}) activity in the presence of 5 and 2 mM $HgCl_2$ respectively.

Presence of $MnCl_2$ enhanced the agarase enzyme activity drastically and significantly ($p<0.05$), of all the three strains. *F. yaeyamensis* agarase exhibited an increase of 262.96% in its activity, *i.e.* the specific activity increased from 0.71 U mg^{-1} to 1.59 U mg^{-1} in the presence of 5 mM $MnCl_2$ and with 2 mM $MnCl_2$, 168.18% hike in activity was observed (1.28 U mg^{-1}). On comparing *A. marinus* and *A. taiwanensis*, agarase from the former was found to be more influenced by Mn^{2+} ions with an activity rise by 262.96% (0.98 U mg^{-1}) at 5 mM and 188.89% (0.78 U mg^{-1}) at 2 mM concentrations. The latter exhibited a respective increase by 150% (0.67 U mg^{-1}) and 97.06% (0.85 U mg^{-1}) in the presence of 5 and 2 mM $MnCl_2$.

Agarases from all the three agarolytic strains exhibited highest activity at 250 mM NaCl concentration except for *F. yaeyamensis* which had the highest activity with 100 mM Na^+ (0.57 U mg^{-1}) concentration (Fig. 2e). Agarases from *A. marinus* and *A. taiwanensis* had a respective loss of 48.65% (0.19 U mg^{-1}) and 32.56% (0.27 U mg^{-1}) activity from its highest activity in the presence of 1000 mM NaCl. Their highest activities were 0.37 U mg^{-1} and 0.40 U mg^{-1} observed at 250 mM Na^+ concentration. *F. yaeyamensis* agarase lost 33.33% (0.44 U mg^{-1}) of its highest activity at 1000 mM Na^+ concentration. Thus, among the three strains, *A. marinus* agarase was found to be more susceptible to higher NaCl levels.

Presence of $CaCl_2$ exhibited an appreciable increase in the enzyme activity (Fig. 2f). Agarases derived from *F. yaeyamensis, A. marinus* and *A. taiwanensis* had a respective rise of 19.53, 37.23 and 55.49% activity at 100 mM concentration. *F. yaeyamensis* agarase was found to be the least influenced by $CaCl_2$.

Effect of β-mercaptoethanol, EDTA and PMSF on agarase activity

The inhibitory effect ($p<0.05$) of mercaptoethanol on activity of agarases from all the three strains is clearly evident from Fig. 3a. Unlike others, *F. yaeyamensis* agarase retained 13.46% of its activity at 5 mM concentration. At 2 mM level, agarases from *F. yaeyamensis, A. marinus* and *A. taiwanensis* had a respective activity loss of 77.88, 88.61 and 89.09%. Thus, *F. yaeyamensis* was observed to be comparatively less affected by mercaptoethanol.

Similar to mercaptoethanol, the chelating agent EDTA was also found to have a significant ($p<0.05$) inhibitory effect on agarase activity (Fig. 3b). Agarases from *F. yaeyamensis* (75.96%) and *A. marinus* (75%) were inhibited to almost similar extent by 5 mM EDTA while at 2 mM level, 68.27 and 71.67% reduction in activity was noticed respectively. *A. taiwanensis* was found to be most affected with a loss of 87.5% activity at 5 mM concentration and 78.13% reduction at 2 mM concentration.

Agarases from all the three agarolytic strains under this study were adversely affected by the presence of the protease inhibiting enzyme PMSF. More than half of the activity was lost by the presence of PMSF in both *F. yaeyamensis* (55.56, 61.11%) and *A. marinus* (57.38, 60.66%) at both 2 and 5 mM concentrations, respectively. *A. taiwanensis* agarase was found to be the least affected, with only 34.04% of activity lost at 5 mM and 10.64% at 2 mM PMSF.

Discussion

Marine microorganisms offer a diverse array of novel biomolecules including degradative enzymes which play a crucial role in carbon nutrient cycling (Pluvinage *et al.*, 2013). Agarases are specific glycoside hydrolases (GHs) which can hydrolyse agar and form oligosaccharides of therapeutic and industrial relevance. Thus, agarases possess a wide array of potentials which needs to be exploited effectively. This study forms the first report on the optimisation of bacterial agarases from *F. yaeyamensis*, *A. taiwanensis* and *A. marinus*. The significance of these strains lies in their source of isolation as coral reefs being rather underexplored for their functional potential and the agarases under this study are isolated for the first time from coral reef ecosystems.

The temperature dependence of agarase enzyme is crucial and the optimal activity of most of the reported agarases falls around 38°C (Fu and Kim, 2010) and marine derived agarases usually exhibit instability at high temperatures (Jonnadula and Ghadi, 2011). The agarase derived from the strain *F. yaeyamensis* AM.5A under this study was found stable over a tested temperature range of 20-80°C with highest activity shown at 40°C. Agarases derived from *A. marinus* AM.17.E1 and *A. taiwanensis* A.69.B2 in the present study, exhibited wide temperature tolerance from 20-80°C with peakl activity seen at 50°C. Those agarases having optimal activity above 40°C can be effectively utilised for the industrial production of agaro-oligosaccharides as it falls above the gelling temperature of agar (Ohta *et al.,* 2004) which prevents the solidification of agar (Lin *et al.,* 2012) and facilitates optimal and uninterrupted enzyme reaction. Agarases are usually more efficient in solution than in gel state as compact bundles of gelled agar or agarose hinder the enzyme action (van der Meulen *et al.,* 1974). Initial stages of digestion usually demand an elevated temperature, as the gelling of the agar can strongly retard the action of agarase (Morrice *et al.,* 1983). Loss of agarase activity at higher temperature can be attributed to the denaturation of the enzyme which in turn influences the enzyme-substrate complex formation (Wang, 1999).

Flammeovirga sp. exhibit strain dependent temperature tolerance. For *e. g.,* an exo type agarase producing *Flammeovirga* strain reported by Han *et al.* (2016) exhibited thermostability only up to 40°C. As per Han *et al.* (2012), extracellular agarases of *Flammeovirga* genus was stable over a temperature range of 0-50°C. Representatives of agarase from the genus, *Flammeovirga pacifica* WPAGA1 isolated from deep marine sediment, was found to have temperature tolerance from 30-50°C (Hou *et al.*, 2015). Agarase from *Flammeovirga* sp. OC4 had optimal activity observed at temperature 30-50°C (Chen *et al.*, 2016). Jean *et al.* (2009) reported the agarolytic activity of *A. marinus* and *A. taiwanensis*. However, little or no work has been done on the agarases from this genus and therefore, a comparative evaluation of the performances of the *Aliagarivorans* strains with different species of the same genus is not possible Lack of information on the properties of the agarases from *A. marinus* and *A. taiwanensis* emphasises the importance of this work.

Agarase enzyme is highly dependent on pH as it has influence on the ionic state which in turn affects the tertiary structure of the enzyme and hence activity. pH can also alter the active sites and cause irreversible inactivation. Any deviation from the optimum pH can cause enzyme denaturation and

drastic diminutions in enzyme activity (Segel, 1976). Agarase from *F. yaeyamensis* strain AM.5A exhibited a wide pH tolerance from 4.0-10.0 with highest activity recorded at pH 7.0. Most of the marine agarases reported, exhibit optimal activity at neutral pH (Wang *et al.*, 2006) or at a weak alkaline pH (Fu *et al.*, 2008) which corresponds to the weak alkaline nature of natural seawater (pH 7.5) (Long *et al.*, 2010). However, the tolerance of *Flammeovirga* strain MY04 varied over a pH range of 6.0-11.0 (Han *et al.,* 2012). Similar was the case of *Flammeovirga pacifica* WPAGA1 which was found to be stable over a pH range of 5.0-10.0 with optimum being 9.0 (Hou *et al.*, 2015). Agarases of *Flammeovirga* sp. SJP92 isolated by Dong *et al.* (2017) from abalone intestine exhibited its optimal activity at pH 8.0 (Dong *et al.*, 2017). *Flammeovirga* sp. OC4 strain exhibited agarase activity at an even lower pH of 3 to the highest of pH 10, with optimal activity recorded at a pH 6.5 (Chen *et al.*, 2016).

Both the strains of *Aliagarivorans* had a wide pH tolerance from 4.0-10.0 with optimum at pH 7.0. Though information on pH tolerance of agarases from *Aliagarivorans* sp. are not available, a closer relative, *Agarivorans* sp. HZ105 (Hu *et al.*, 2008) was reported to produce extracellular beta agarases with pH range from 6.0-10.0. Agarase from *Agarivorans albus* YKW-34, also had a wide pH range of 6.0-9.0 (Fu *et al.*, 2008) with optimum activity seen at pH 8.0. Close to our strains *A. marinus* AM.17.E1 and *A. taiwanensis* A.69.B2, *Agarivorans* sp. JAMB-A11 exhibited its optimal activity at pH 7.5-8.0 (Ohta *et al.*, 2005).

Time course of enzyme reaction is another critical factor for optimisation of reaction products. For all the three strains under this study, a time period of 45 min was found sufficient for optimal activity. Complete purification of the enzyme might further reduce the reaction time (Segel, 1976). In addition to temperature, pH and time, enzyme stability is also influenced by other factors such as chemical nature of buffer, concentration of various preservatives and concentration of metal ions (Segel, 1976). Metal ions have been proved to have a significant influence on the activity of agarase enzyme (Suzuki *et al.,* 2003). The abundance of metal ions in seawater *viz,* Na^+, K^+, Ca^{2+} and Fe^{3+} can be a contributing factor. However, the effect of metal ions on agarases varies with bacterial species. For instance, agarases from *Saccharophagus* sp. AG21 (Lee *et al.*, 2013) was found to be inhibited by Mn^{2+}, while all the three agarolytic strains in this study showed a positive response in presence of Mn^{2+}. The underlying mechanism behind these varying responses needs to be elucidated for manipulating its activity for industrial applications. The effect of divalent ions on agarase enzyme was also found to be varying with the biochemical nature of the enzyme. $MnCl_2$ and $CaCl_2$ were found to have a boosting effect on the agarases from all the three agarolytic strains. The inhibition of activity by $HgCl_2$ and $CuSO_4$ indicated the presence of SH group in the active site of the enzyme which undergoes oxidation in the presence of these ions. Since Agarase from *F. yaeyamensis* remains unaffected by the presence of $CuSO_4$, it indicates the absence of -SH group in its active site. Furthermore, $HgCl_2$ and $CuSO_4$ can form complexes with the enzyme, thereby preventing it from product formation. But in contradiction, *F. yaeyamensis* was found to be inhibited by $HgCl_2$ but not by $CuSO_4$. Further molecular level investigations are needed for a detailed explanation. Effect of metal ions on agarase activity is strain dependent. For *e. g.* K^+ ions hindered the agarase activity of all the three strains under this study, whereas it had a stimulatory effect on *Bacillus* sp. MKO3 strain (Suzuki *et al.,*2003; Gupta *et al.*, 2013). However, the effect of Ca^{2+} (stimulating) as well as $Hg^{2+}$and Cu^{2+} ions (detrimental) were similar to that of the agarases from the three strains under the present study. Similarly, GH16 beta-agarases from *Vibrio* sp. strain V134 (Zhang and Sun, 2007) was found to be positively affected by NaCl and KCl unlike the strains used in the present study.

Usually bacterial enzymes including agarases require only low NaCl concentrations for their optimum activity. Agarases from all the three strains investigated in this study exhibited a declining trend in activity with rise in NaCl concentrations. A slight increase in activity was observed at 250 mM for *Aliagarivorans* strains after which it decreased. Beta agarase II of *Pseudomonas atlantica* (Morrice *et al.*, 1983) and that of *Pseudomonas* sp. strain W7 (Ha *et al.*, 1997) had optimum activity at NaCl concentrations of 150 and 900 mM respectively. But, *Microbulbifer thermotolerans* JAMB-A94 with a thermo stable beta agarase (Takagi *et al.*, 2015) exhibited similar level of activity with NaCl concentrations up to 1250 mM, after which the activity decreased with further increase in concentration. Among the agarases studied here, only *F. yaeyamensis* agarase exhibited highest activity at 100 mM, while agarases from the other two strains had peak activity at 250 mM NaCl concentration. As per Han *et al.* (2012), extracellular agarases of *Flammeovirga* genus are stable over a wide range of NaCl concentrations (0 - 0.9 mol l^{-1}).

Flammeovirga sp. strain MY04 was found to be inhibited by Ca^{2+}, Fe^{2+} and Mn^{2+} (Han *et al.*, 2012). This was in contradiction to the response of *F. yaeyamensis* AM.5A towards Ca^{2+} and Mn^{2+} in this study. Calcium ions were found to stimulate the enzymatic activity. For the range of concentrations tested from 0-100 mM, the activity was found to increase with increase in Ca^{2+} concentrations.

The negative effect of EDTA on agarases from *Pseudoalteromonas* sp. CY24 and *Vibrio* sp. F6 has been reported (Ma *et al.*, 2007). *Bacillus* sp. H12 agarase was also inhibited by the chelating agent, EDTA at 2 and 5 mM concentrations. The α-neoagaro-oligosaccharide produced by *Bacillus* sp. MKO3 was inhibited by EDTA at 2 mM level (Suzuki *et al.*, 2003). In contrast, the agarase activity from *A. albus* YKW-34 was not affected by EDTA (Fu *et al.*, 2008), whereas that from *Bacillus* sp. MK03 (Suzuki *et al.*, 2003) was significantly enhanced by EDTA. However, agarases from all the three test strains under this study exhibited an inhibitory effect in the presence of EDTA. Probably, due to the chelating effect of EDTA, they form complexes with the ions in the enzyme active site causing inhibition of enzyme activity. Since the agarase enzyme is dependent on metal ions as cofactors, they can be referred to as metalloenzymes, but not all agarases are metal dependent.

Inhibition of enzyme activity by the reducing agent, mercaptoethanol reveals the presence of -SH group in or near the active site of the enzyme. The reducing compounds aid the SH group to be stable. Agarase activity of all the three strains in this study were significantly ($p<0.05$) reduced when incubated in the presence of 2 and 5 mM mercaptoethanol. The agarase produced by *Bacillus* sp. MKO3 remained active even in the presence of 2 mM β-mercaptoethanol (Suzuki *et al.*, 2003), whereas agarase activity of *Acinetobacter junii* PS12B was enhanced by mercaptoethanol, while EDTA had an inhibitory effect (Timoty *et al.*, 2016).

Agarases belonging to the serine enzyme category will be inhibited by the presence of PMSF, a serine inhibitor. Since all the three strains exhibited an abrupt decrease in activity, it clearly indicates that the agarases have serine residues in their active sites. *Flammeovirga* sp. strain MY04 was reported to be inhibited by EDTA and PMSF (Han *et al.*, 2012).

Results of the present study indicated no relation between the optimal growth conditions of the bacterial strains with that of the optimal conditions for peak agarase activity. The effect of metal ions on agarases depends largely on the biochemical properties of the enzyme. Agarases are characterised into two groups *viz,* α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81), each having unique hydrolysis pattern. Majority of the reported agarases belong to the beta group (Fu and Kim, 2010). In general, β-agarases can be divided into two types (type I and type II) according to their substrate specificity and product profile. β-agarase I has a molecular weight of 32 kDa and activity was observed over a pH range of 3.0-9.0 with optimum being 7.5. β-agarase I can be stimulated by NaCl optimally in the range of 100-200 mM (Fu and Kim, 2010). Alkali earth metals such as $CaCl_2$ and $MgCl_2$ were also found to have a stimulating effect (Morrice *et al.*, 1983). Whereas β-agarase II has a lower ionic strength, but active at NaCl concentrations above 100-200 mM (Morrice *et al.*, 1983). Its activity was observed over a pH range of 6.0-9.0 with optimum being 7.5. Comparatively slighter enhancement in activity was observed with $CaCl_2$ and $MgCl_2$ (Morrice *et al.*, 1983). The properties of all the three agarases discussed under this study similarises with that of the β-agarases type II.

All the three strains of agarolytic bacteria investigated in this study were found to be potential sources of agarase enzyme, which offer reliable candidates for industrial applications. However, a significantly broad temperature range of activity was exhibited by agarase from the strain *A. marinus* facilitating its easy manipulations at varying temperatures. Further investigations on genes encoding the agarase enzyme, its pattern of hydrolysis, as well as the characteristics of hydrolysis products are important for understanding its potential as a promising catalyst in large scale applications. No information is so far available on the optimisation of agarases from the bacterial species under this study and hence, a comparative analysis of the enzyme activity with that of the same species is rather not feasible. Despite the high catalytic activities, only a minor fraction of marine microbial agarases have been successfully used in industrial applications. Behavioural pattern of agarases at different physical and chemical environment are very much essential to assess the suitability of these enzymes in large scale applications. Developments concerning the use of these agarases in the biotechnological industry can be expected only if vast and in-depth studies on these enzymes are undertaken. For the same reason, this work can be a contribution to the knowledge on marine agarases from these underexplored agarolytic strains.

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