CHAPTER 6

DISCUSSION
This chapter deals with the discussions related to results of experimental work undertaken during studies on screening and identification of agarolytic bacteria. The selection of strain CMC-5 for further studies was based on its property of multiple polysaccharide degradation as well as its capacity to degrade thalli of seaweeds. On the basis of polyphasic studies strain CMC-5 found to be closely related to *Microbulbifer*. Optimization of culture conditions was achieved which were later used for large scale culture of strain CMC-5. The purification of agarase enzyme was achieved by combination of ion exchange and gel filtration. The biochemical properties of 59 kDa purified agarase were studied. Finally, potential of agarase enzyme as producer of oligosaccharides with antioxidant property, high molecular weight DNA recovery from agarose gel and its role in preparation of single cell detritus were investigated.

6.1 SCREENING FOR AGAR DEGRADING BACTERIA:

The marine ecosystems with diverse niches are rather unexplored sources of bacterial enzymes which can be exploited for novel catalytic and therapeutic activities. A focused attention must be paid to biochemical properties of enzymes if they have to be used on an industrial scale.

Two different methodologies for screening agarolytic bacteria were adopted. Direct plating of the water samples resulted in isolation of the total bacteria which is an exact reflection of bacterial isolates present in the environment. Enrichment technique generally results in the isolation of dominant bacterial isolates which might over grow other possible slow growing potential strains and thus during subsequent enrichment process would result in elimination of that potential bacterial strains (Jannasch, 1967).
During the current investigation, ASW medium was used for isolation of agarolytic bacteria. In marine ecosystems, seaweed cell walls and other potential sources of polysaccharides, which are target for microbial action are naturally present as a complex consortia or mixture of polysaccharides. Thus, in order to investigate whether any other polysaccharide degrading property can be co-associated with agarolytic property, ASW agar plates supplemented with other polysaccharides were used during primary screening. Thus, ASW agar, ASW agar+CMC and ASW agar+alginate plates were used for screening purpose.

As observed from the screening results, many bacterial isolates obtained from direct plating of coastal water/sediments resulted in isolation of many bacterial isolates. The colony morphology of these isolate were different to those obtained by enrichment technique. Although the bacterial isolates obtained by direct plating and enrichment method could grow on their respective polysaccharide/s plates, none of these isolates showed any clearance zone/pits around the colonies. After 2-3 subcultures, few of these isolates failed to grow on respective polysaccharide/s plates. The other bacterial isolates which showed growth on polysaccharide/s plates after repeated subcultures did not show clearance zone or pit formation and presumably degraded polysaccharides by the exo-mechanism. This was proven by the fact that clearance was visible only when lugol’s iodine specific for detection of agar degradation was spread, a clearance zone was observed (Hodgson and Chater, 1981). An exo-acting polysaccharase have limited ecological significance in comparison to endo-acting polysaccharases and are needed at the end of the degradation cycle. Hence,
these bacterial isolates were not selected for further screening. Similarly no promising agarolytic bacteria were isolated from water samples collected from mangrove area.

The cell walls of seaweeds offer a unique micro-niche in the form of polysaccharides for carbon mineralization. The cell walls of brown seaweeds contain predominantly alginic acid whereas agar and carrageenan are predominant in cell wall of red seaweeds (Kloareg and Quatrano, 1988). Complex bacterial communities have been found to be associated with seaweeds. Bacteria belonging to different genera such as *Sulfitobacter*, *Halomonas*, *Cinetobacter*, *Planococcus*, *Arthobacter* etc., existed in abundance on the surface of brown and red seaweeds. Further microflora compositions of two species of brown algae were observed to be different (Beleneva and Zhukova, 2006).

In our present study, when fresh seaweeds were used as a resource for bacterial screening by direct plating or enrichment, a white matt growth of bacteria was observed on all polysaccharide/s plates. Direct plating of water sample from decomposing seaweeds resulted in isolation of many bacteria colonies on ASW agar, ASW agar+CMC and ASW agar+alginate plates. A total of 22 bacterial isolates were picked from the above polysaccharide/s plates as these isolates showed clearance zones as well as deep craters. However enrichment of bacterial population from decomposing seaweeds resulted in bacterial isolates which formed small, white colour colonies and did not depict craters or clearance zone. Thus, polysaccharide degrading isolates which were prevalent in decomposing seaweeds (as observed in direct plating)
probably have been overgrown and eliminated by the small white colored colonies which were the predominating isolate obtained during enrichment.

Although polysaccharide degrading bacteria have been isolated from diverse niches such as coastal water, sediments, rhizosphere, the present study indicates that polysaccharide degrading bacteria predominantly associated with decomposing seaweeds could be successfully isolated. Thus the polymer aggregates of the seaweed cell wall are hotspots for bacterial associations. Utilization of these polysaccharides through carbon mineralization is largely dependent on bacterial action leading to recycling of organic matter in marine environments.

6.2. STRATEGY FOR DETECTION OF MULTIPLE POLYSACCHARIDE DEGRADATION:

Polysaccharide degradation can be easily monitored by observing the growth of bacteria on solid medium plates containing homologous polysaccharides. However, presence of organic impurities such as proteins and other carbon compounds are very common in polysaccharide preparations and growth on polysaccharide plates would enable non-polysaccharide degraders to grow utilizing this contaminating proteins or impurities as carbon source, resulting in false positive results. During preliminary screening for agar degrading bacteria, ASW agar, ASW agar+CMC and ASW agar+alginate plates were used for screening agarolytic bacteria. The assumption was that presence of additional polysaccharides in plates besides agar would promote selection of other polysaccharide degrading trait along with agarolytic property. CMC
and alginate were chosen as polysaccharides of choice as they are prevalent dominantly in red and brown seaweeds.

Agar is the common solidifying agent used in all three selective plates. Therefore a reliable method for the detection of individual polysaccharide degradation was required to enable the identification of exact polysaccharide degrader. A number of chemical assays have been described in literature. Dye based plate screening methods are essentially based on the principle that polysaccharides interact non covalently with dye in the area where polysaccharide degradation occurs resulting in clearance zone in contrast to the dark polysaccharide-dye complex formation where no degradation is evident. Plate assay using chromogenic substrates are also often used to screen potential polysaccharide degrading strains (Barasan et al, 2001). However, due to the exorbitant cost of chromogenic substrates, dye based identification is more economical and commonly used.

Congo red interacts with polysaccharides containing (1,4)-β-D-glucans, (1,3)-β-D-glucans, (1,4)(1,3)-β-D-glucans and (1,4)-β-D-xylans. Congo red has been commonly used for the identification of cellulolytic bacteria from rumen and soil samples as well as for zymogram studies of xylanases (Teather and Wood, 1982; Hendriks et al, 1995; Breccia et al, 1995). In the present study, congo red was used for identification of CMCase, xylanase and chitinase activities of the strains by growing them in ASW agar, ASW agar+CMC, ASW agar+xylan and ASW agar+chitin plates respectively. As a consequence of polysaccharide degradation by the strains, light coloured clearance zone was generated around the colonies after spreading congo
red. The clearance zone around the colonies could be intensified after flooding with 1 M NaCl solution. The clarity of the clearance zone was further enhanced by washing with 1M acetic acid (Hendricks et al, 1995). Congo red did not show any clearance zone when ASW agar alone was used, indicating the clearance zone observed on ASW agar+CMC, ASW agar+chitin and ASW agar+xylan were specific to degradation of cellulose, chitinase and xylanase respectively.

Agar degrading bacteria were routinely detected on ASW agar plates by flooding lugol’s iodine solution as reported by Gran in 1902. Since then, this method is widely used for the detection of agarase producing and agarase mutant strains. Development of light yellow colour next to the agarolytic bacterial colony indicates complete loss of integrity in agar polysaccharide structure and partial degradation of agar polysaccharide. The undegraded agar stains dark brown. It is believed that the failure to form the deep brown colour is due to loss of double helical structure of agar polysaccharide due to action of agarase enzyme (Hodgson and Charter, 1981).

Cetylpyridinium chloride (CPC) is a cationic polymer known to react with highly sulphated galactans resulting in precipitation of polysaccharide. Gacesa and Wustman (1990) detected alginase activity on media plate containing alginate by spreading the cetylpyridinium chloride. Staining with ruthenium red followed by treatment with 95% alcohol, diluted HCl and CaCl₂ have been also been reported in literature for detection of alginase lyase activity on media plates containing alginate (Wong et al, 2000). Similarly, CPC has been used for detection of carrageenase activity on media plates as well as for zymogram studies of carrageenase as
carrageenan contains highly sulphated galactans in its structure (Ohta and Hatada, 2006). Alginolytic and carrageenolytic nature of polysaccharide degrading bacteria were detected by growing on ASW agar+alginate and ASW agar+carrageenan plates after which CPC was added. CPC did not precipitate ASW agar plates, indicating it is specifically detects alginolytic and carrageenolytic activity.

During the present investigation, agar has been proved to be suitable for detecting the multiple polysaccharase activities on plates containing any other polysaccharides. Gelrite, another solidifying agent is widely used as it is resistant to microbial decomposition (Shungu et al, 1983). Gelrite is a linear polysaccharide containing repeat unit of tetrasaccharide -(3-β-D-GlcA-(1,4)-α-D-Glc-(1,4)-α-D-Glc-(1,4)-α-L-Rhap-1)- containing glucuronic acid, glucose, rhamnose and O-acetyl moieties. It forms a firm gel in the presence of divalent cations such as Mg$^{2+}$ or Ca$^{2+}$ at a concentration of 2mM. Similarly, 100 mM Na$^+$ and K$^+$ form firm gel with gelrite (Kelco application bulletin). In the present study, ASW was used as basal medium and contain high molar concentration of Mg$^{2+}$ and Na$^+$ ions. Addition of gelrite to ASW results in instantaneous solidification which cannot be controlled even at high temperatures. Hence gelrite was not preferred for preparations of polysaccharide/s plates. Screening for CMCase, xylanse, chitinase, alginase and carrageenase activities with various chromogenic/precipitating agents in the presence of agar showed that agar polysaccharide is not interfering with the staining capability of the respective chromogenic/precipitating agents as mentioned.
6.3 POLYSACCHARASE ACTIVITIES OF BACTERIAL ISOLATES:

Based on secondary screening (as mentioned in section 6.2) 15 bacterial isolates were found to be multiple polysaccharide degraders and degraded more than three polysaccharides (Table 3.3). Further, 15 bacterial isolates (AA-1 to AA-7 and CMC-1 to CMC-8) obtained were tested for production of polysaccharases by growing in ASW broth containing 0.2% of agar, alginate or CMC. Isolates CMC-3, CMC-4, CMC-5 and CMC-8 were observed to show high polysaccharase (CMCase, agarase and alginase) activities compared to other bacterial strains. Further, in vitro thalli degradation studies with respect to 15 isolates, indicated that strain CMC-2, CMC-3, CMC-5 and CMC-6 degraded seaweed thalli and released single cells. On the basis of multiple polysaccharide degradation studies, polysaccharase activity in culture supernatant and in vitro thalli degradation, bacterial isolate CMC-5 was found to be superior in all the three criteria and hence selected for further study.

Strain CMC-5 was isolated from decomposing seaweeds and degraded 6 different polysaccharides, namely agar, CMcellulose, alginate, carrageenan, xylan and chitin. Polysaccharide utilization test also revealed that strain CMC-5 not only degraded polysaccharides but also was capable of utilizing these six different polysaccharides as evident by bacterial growth estimated at A600. The ability to degrade multiple polysaccharides has been reported in Saccharophagus degradans 2-40 and Microbulbifer elongatus. Saccharophagus degradans 2-40 was isolated from decaying salt marsh and found to degrade 10 different polysaccharides (Ensor et al, 1999). An unknown unique bacterium degrading seven different polysaccharides was obtained.
from *Fucus disticus* (Quatrano and Cladwell, 1978). Thus microbial community associated with different biota especially decomposing seaweeds seems to be a potential niche for isolating multiple polysaccharide degrading bacteria.

However, besides the marine sources as mentioned above, even non-marine sources such as terrestrial rhizosphere have served as source for isolating several strains of *Paenibacillus* sp which showed multiple polysaccharide degrading activity (Hosoda et al, 2003; Li et al, 2003). Similarly, several strains of *Microbulbifer* degrading multiple polysaccharides have been reported from various niches (Gonzalez et al, 1997; Tanaka et al, 2003; Yoon et al; 2003b; Yoon et al, 2004; Wang et al, 2009).

### 6.4 IDENTIFICATION OF STRAIN CMC-5:

Strain CMC-5 was gram negative, non motile, aerobic, grew optimally at 30°C and required growth medium to be amended with 2-3% NaCl for optimum growth. Since strain CMC-5 is non-motile. Requirement of NaCl in the medium for growth suggest strain CMC-5 is a marine bacteria. Biochemical studies of strain CMC-5 did not offer any possible clue for identification of bacteria. The biochemical features of strain CMC-5 was compared with other known bacteria by using the software for Probabilistic Identification of Bacteria for Windows (PIBWin) Ver 1.9.2. (http://www.som.soton.ac.uk/staff/tmb/pib.htm). The strain CMC-5 was observed to show similarity with the genus *Pseudomonas*.

Strain CMC-5 was observed to utilize large number of substrates, majority of which are either simple soluble sugars or intermediates of various biochemical cycles.
that few of the carbon compounds which are utilized by strain CMC-5 such as glucosamine, cellobiose, galactose etc are structural components of polysaccharides. The antibiotic profile of strain CMC-5 as reported in table 3.5., indicates resistance to most commonly used antibiotics. Chemotaxonomic approach was undertaken to identify the strain CMC-5 on the basis of FAME analysis. Cellular fatty acid methyl esters analyzed by gas chromatography is commonly used for bacterial identification (Able et al, 1963; Eder, 1995). Application of this method is well adopted for the identification and classification of bacterial species (Welch, 1991). The FAME profile of strain CMC-5 was compared with internal reference library, which was available with the chromatography system. The FAME profile of strain CMC-5 failed to match with any of the bacterial species listed in the internal reference library. It was observed that strain CMC-5 has relatively high concentrations of following fatty acids: iso-C15:0, iso-C17:1o9c, iso-C17:0, iso-C11:3-OH, C18:1 o7c, iso-C11:0 and C16:0. Concentrations of other fatty acids observed in FAME profile of strain CMC-5 were less than 5%. In addition, levels of C16:1 o7c, iso-C15:0, 2-OH, C19:1o6c and C19:0cyclo fatty acids were not determined due to poor resolution of the chromatography system (Table 3.5.).

FAME profile of strain CMC-5 was compared with other bacterial species. The FAME profile of strain CMC-5 showed similarity with Microbulbifer genus. Microbulbifer which was proposed by Gonzalez et al, for rod shaped, strictly aerobic marine bacteria belong to Proteobacteria (Gonzalez et al, 1997). Iso-C15:0 is reported as major fatty acid in strain CMC-5. The bacterial species which are included into Microbulbifer genus also contains iso-C15:0 as major fatty acid (Yoon et al, 2003a&b).
The other major genus *Pseudomonas* which also belongs to *Proteobacteria* contains C\(_{16:0}\) as major fatty acid. The genus *Alcanivorax* contains C\(_{18:1\,\alpha7c}\) and C\(_{16:0}\) as major fatty acids (Fernandez-Martinez et al, 2003). Thus, the strain CMC-5 is reasonably closer to the genus *Microbulbifer* based on FAME analysis of cellular fatty acids.

On the basis of PIBwin software, strain CMC-5 was observed to be similar to *Pseudomonas* on basis of biochemical tests. However, FAME analysis of strain CMC-5 indicates a possible affiliation to the genus *Microbulbifer*. It is important to note that, the genus *Microbulbifer* was reclassified from the genus *Pseudomonas* based on the presence of iso-C\(_{15:0}\) as major fatty acid and predominant respiratory ubiquinone-8 (Q-8). The authentic *Pseudomonads* contain C\(_{16:0}\) as major fatty acid and ubiquinone-9 (Q-9) (Oyaizu and Komaguta, 1983; Yoon et al, 2003a&b). Reclassification of *Pseudomonads* based on 16S rDNA sequence also differentiates the genus *Microbulbifer* from *Pseudomonas* (Anzai et al, 2000). Thus, on the basis of isoC\(_{15:0}\) as major fatty acid in strain CMC-5 and low levels of C\(_{16:0}\) fatty acid clearly indicates that strain CMC-5 is more closely related to *Microbulbifer* genus than the *Pseudomonas* genus.

A total of 1517 bases of 16S rDNA sequence of strain CMC-5 were determined. The sequences homologous to 16S rDNA sequence of strain CMC-5 were retrieved from RDP database (Ribosomal Database Project, http://rdp.cme.msu.edu). The homology search at RDP indicated that the sequence of strain CMC-5 was homologous to the members of gamma subclass of *Proteobacteria* and in particular to the genus *Microbulbifer*. Multiple sequence alignment of 16S rDNA sequence was achieved.
using Clustal W program (Thompson et al, 1994). Phylogenetic trees were constructed by tree making algorithms such as the neighbour-joining, maximum-likelihood and maximum-parsimony (Felestein, 2006).

The phylogenetic tree constructed using maximum-likelihood algorithm indicated that this strain formed a coherent cluster with the clade that comprises type strains of *Microbulbifer* genus by a bootstrap confidence level of 100%. It shared a similarity of 99% with *M. elongates* DSM 6810 (AF500006), 98% with *M. salipaludis* (AF479688), 97% with *M. hydrolyticus* IRE-31 (U58338) and 95% with *M. maritamus* (AY377986). Similar tree topology was observed when trees were constructed using neighbour joining and maximum parsimony algorithms. As apparent from phylogenetic tree, strain CMC-5 closely resembles *Microbulbifer elongatus* DSM 6810T.

Although, the strain CMC-5, shares 99% similarity with *M. elongatus* DSM6810T (AF500006) and close similarity with other species of *Microbulbifer*, there are note-worthy differences in biochemical properties. The differences in the biochemical characteristic features between strain CMC-5 and *M. elongatus* DSM6810T are shown below (Table 6.1.).
The strain CMC-5 is non motile and did not show H$_2$S production when compared to *M. elongatus* DSM6810$^T$. Strain CMC-5 was isolated from decomposing
seaweeds and forms cream colour colonies on Zobell marine agar plates whereas *M. elongatus* DSM6810\(^T\) has been isolated from intertidal bottom sediments and forms yellowish orange colour colonies on Zobell marine agar (Yoon et al, 2003a). Similarly, the average size of strain CMC-5 is in same range as reported for most *Microbulbifer* strains whereas the cells of *M. elongatus* DSM 6810\(^T\) are larger (Yoon et al, 2003a&b., Yoon et al, 2004). Also in comparison to *M. elongatus* DSM 6810\(^T\), strain CMC-5 utilize glucose, galactose and inositol and does not utilize sucrose. Besides variation in biochemical properties as shown in Table 6.1., the striking difference was degradation of xylan by strain CMC-5 whereas *M. elongatus* DSM 6810\(^T\) did not degrade xylan (Yoon et al, 2003a).

The G+C mol % content of strain CMC-5 was estimated as 65.6 by thermal denaturation method (Fig 3.6). On an average, the G+C mol% content for *Microbulbifer* has been reported to be 59. The %G+C content for *M. Hydrolyticus*, *M. salipaludis*, *M. maritamus*, and *M. donghaiensis* was 58, 59, 57.7 59.9, and 57.8% respectively (Gonzalez et al, 1997; Yoon et al, 2003a&b; Yoon et al, 2004; Wang et al, 2008). However, recently G+C mol% content in *Microbulbifer halophilus* has been reported as 63.2 which is closer to the %G+C content reported for strain CMC-5 (Tang et al, 2008).

All these differences clearly indicate that strain CMC-5 is morphologically and biochemically different from *M. elongatus* DSM6810\(^T\) strain. Hence the strain CMC-5 can be referred as morphovar and biovar of *M. elongatus* DSM6810\(^T\). Based on results
obtained by fatty acid methyl ester analysis and 16S rDNA study, strain CMC-5 has been classified as

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The strain CMC-5 will be hence referred as *Microbulbifer* strain CMC-5. The Microbulbifer strain CMC-5 has been submitted to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh and the accession is MTCC 9889.

6.5 EFFECT OF NITROGEN AND CARBON SOURCES ON AGARASE PRODUCTION:

The effect of nitrogen and carbon source on production of agarase enzyme was studied. Microorganisms require soluble nitrogen source for their growth and essentially for protein synthesis. Few reports indicate that nitrogen sources influence agarase enzyme production. Lakshmikanth et al, (2006b) reported enhanced production of agarase enzyme by *Acinetobacter* sp. AG LSL-1 in the presence of various nitrogen sources. Sodium nitrate could induce 0.41 U/ml of agarase enzyme, whereas ammonium nitrate and yeast extract induced 0.31 and 0.25 U/ml of agarase enzyme respectively. Similarly, addition of yeast extract to culture medium led to increased production of
agarase enzyme from *Pseudomonas aeruginosa* AG LSL-11 (Lakshmikanth et al, 2006a). Effect of various individual amino acids on agarase production in *Cytophaga flevensis* was studied. Casamino acids showed highest production of agarase enzyme (Van Der Meulen and Harder, 1976). It is assumed that organic nitrogen sources can easily replenish the existing internal pool of amino acids within the cell thus facilitating the availability of amino acids for the protein synthesis. Induction of a novel agarase enzyme 0072 from *Vibrio* sp. JT0107, was observed when grown in presence of agar supplemented with polypeptone (Sugano et al, 1995). Thus, the presence of nitrogen supplements can increase the overall yield of various agarase enzymes.

Strain CMC-5 did not require any vitamins or growth factors for growth as well as for production of agarase enzyme. In the present study effect of various nitrogen sources such as yeast extract, peptone, casamino acids and ammonium chloride on production of agarase enzyme was undertaken. The strain CMC-5 showed highest production of agarase enzyme when grown in agarose with 0.05% yeast extract in contrast to agarase production when peptone and casamino acids were used as nitrogen source. This may be due to the fact that yeast extract is rich in B-complex vitamins, amino acids, peptides and carbohydrates. Since they are in water soluble form, they can be easily assimilated by strain CMC-5 resulting in rapid growth and induction of agarase enzyme. Peptone and casamino acids are acid hydrolysates of proteins generally milk protein and are rich in amino acids. Metabolic consumption of amino
acids is relatively energy consuming process to the bacteria and thus show low levels of induction in comparison to level of agarase production when yeast extract was used.

Type of carbon sources also plays a vital role in the production of enzymes from bacteria. Agar is degraded by enzymatic activity into oligosaccharides. These oligosaccharides are further degraded to release soluble sugars, which are easily utilized by bacteria. Soluble simple sugars generally cause catabolite repression in bacteria, which is an important phenomenon in the regulation of enzyme synthesis. Catabolite repression is a genetic regulation mechanism in bacteria, where the accumulated metabolic products represses the synthesis of enzymes responsible for the formation of metabolic products. Catabolite repression by glucose in agarase enzyme production has been studied in *Streptomyces coelicolor* A3(2). It was found that enzyme responsible for glucose phosphorylation plays a major role in catabolite repression at transcription and inducer exclusion levels (Kwakman and Postma, 1994). Induction of agarase enzyme was observed in *Cytophaga flevensis* in the presence of different simple sugars. D-xylose induced highest levels of agarase enzyme compared to D-galactose, which is a major structural unit of agar polysaccharide (Van Der Meulen and Harder, 1976). Similarly, in *Saccharophagus degradans*, a multiple polysaccharide degrading bacteria, polysaccharide degrading enzymes were repressed in the presence of glucose whereas xylose and galactose were able to induce low levels of agarase enzyme (Ensor et al, 1999). In the present study, no agarase production was observed when strain CMC-5 was grown in glucose or galactose as sole carbon sources. However levels of agarase production in the presence of cellobiose, ZMB,
maltose, glycerol and succinate were 63, 40, 40, 12 and 10.6% respectively. Induction of polysaccharase synthesis by monomeric and dimeric substrate is commonly observed in bacteria (Miyazaki et al, 2005). Besides agar, cellobiose and maltose was also observed to produce agarase in strain CMC-5. This might be due to structural similarity of cellobiose and maltose with neoagarobiose, which is a known inducer for agarase enzyme (Whiteland et al, 2001). The failure to produce agarase enzyme by strain CMC-5 especially by glucose is due to catabolite repression as reported for other agarase enzymes. The inability to produce agarase enzyme in presence of glucose due to catabolite repression is well documented for S. coelicolor (Kwakman and Postma, 1994). Similarly other agarolytic bacterial strains such as Cytophaga flevensis, Alteromonas strain 049/1, Pseudoalteromonas antarctica N-1, Saccharophagus degradans 2-40, Pseudomonas aeruginosa AG LSL-11 and Acinetobacter AG LSL-1 showed inhibition of agarase expression due to catabolite repression (Van Der Meulen and Harder, 1976; Agbo and Moss, 1979; Vera et al, 1998; Ensor et al, 1999; Lakshmikanth et al, 2006a; Lakshmikanth et al, 2006b). When the bacteria were grown in glucose or other simple sugars, the cAMP levels are known to be low. A high level of cAMP appears when glucose is depleted or absent in the medium. Binding of cAMP to the catabolite activator protein (CAP) releases the catabolite repression and induces the production of proteins or enzymes responsible for the metabolism (Ensor et al, 1999).

The inhibitory effect on agarase production due to above simple sugar was overcome when the above sugars were co-supplemented with agarose in strain CMC-5.
Similar observations were reported from *Acinetobacter* AG LSL-1 and *P. aeruginosa* AG LSL-11. These strains could produce high levels of agarase enzyme when agar was co-supplemented with other sugars such as fructose, xylose and mannitol (Lakshmikanth et al, 2006a&b, 2006). Similarly, induction of agarase enzyme was observed in *Cytophaga saccharophila* (024) and *Alteromonas* sp. (049/1) when grown in the presence of agar supplemented with glucose (Van Der Meulen and Harder, 1976; Agbo and Moss, 1979).

Since catabolite repression of glucose was overcome on addition of agarose in *Microbulbifer* strain CMC-5, it is believed that although the catabolite repression could inhibit the enzyme synthesis, basal level of agarase enzyme production would lead to the slow degradation of agar resulting in accumulation of oligosaccharides as degradation products. The accumulated oligosaccharides would then act as inducers leading to the increased production of agarase and thus overcoming the catabolite repression. Chitin degradation by *Vibrio furnisii* involved the recognition of chitin oligosaccharides, possibly disaccharide released (GlcNAc)$_2$ which acts as an inducer during chitin degradation (Li and Roseman, 2004). Induction of cellulase was observed in *Hypocrea jecorina* when galactose was provided as sole carbon source. It is believed that D-galactose or galactose bound to galactokinase or D-galactose-1-phosphate is important molecules for the cellulase induction (Karaffa et al, 2006). Similarly, lactose also can induce the cellulase activity in *Hypocrea jecorina* (Seiboth et al, 2005). Sophorose, is known to induce cellulase in many microorganisms such as *Trichoderma* (Loewenberg JR and Chapman CM 2004; Nisizawa et al, 1971). Similarly,
gentiobiose, which is a by-product produced from the cellobiose by the action of β-galactosidase can also induce the cellulase (Kurasawa et al, 1992). Thus simple sugars can be considered as easy and economically feasible sources for the induction and large scale production of polysaccharide degrading enzymes.

In natural environments, microorganisms are generally exposed to various polysaccharides in different combinations. A polysaccharide degrading bacterial consortia would be ideal choice for biodegradation of all these polysaccharides. However, bacteria have evolved to utilize various polysaccharides. Microorganisms like *Saccharophagus degradans* 2-40 and *Clostridium cellulovorans* have developed enzyme systems to degrade different polysaccharides (Ensor et al, 1999; Kosugi et al, 2001). When microorganisms degrade different polysaccharides to respective oligosaccharides, they enter into the cell and induce the expression of enzymes responsible for respective polysaccharide degradation. However, these heterologous oligosaccharides might be structurally related and may cause cross induction of unrelated polysaccharide degrading enzymes. To understand the induction of different polysaccharide degrading enzymes in strain CMC-5, it was grown in various polysaccharide combinations. Agarase enzyme induction was observed in all tested combinations whereas production of other polysaccharase was not observed in all tested combinations. Polysaccharide mix could induce all the five tested polysaccharase activities in the culture supernatant. The induction of agarase in all possible combination of polysaccharides has not been reported anywhere in the literature. As reported previously, cellobiose, a disaccharide has been shown to induce
agarase enzyme in strain CMC-5. It is known that hydrolysis of CMC by CMCase releases cellobiose. The cellobiose released during CMC degradation thus would act as the inducer for the agarase enzyme. Similar reports are available for the induction of CMCase of enzyme from *Penicillium purpurogenum* (Kurasawa et al, 1992). It is obvious that the degradation of complex polysaccharides releases small, medium and large sized oligosaccharides by the action of enzymes based on mode of activity. The release large sized oligosaccharides generally act as signal inducers for the enzymes. It was observed that xylan degrading enzymes can be induced by medium and large sized oligosaccharides than small sized oligosaccharides (Miyazaki et al, 2005). Induction of different xylanase enzymes from *Cellulomonas cellulosorans* was observed in different substrates such as cellulose, avicel, oat spelt xylan and birchwood xylan (Kosugi et al, 2001). Similarly, induction of xylanase enzyme by CMC and CMCase enzymes by xylan substrates are reported widely. This may be due the multiplicity of the enzymes, which was widely reported for xylanase and CMCase enzymes (Wong et al, 1988). Multiplicity of polysaccharide degrading enzymes is a common phenomenon, where the structurally related substrates can be degraded by heterologous enzymes and the degraded products acting as inducers for the production of enzymes. In this present study also, it was observed that xylanase was induced when the strain CMC-5 was grown in CMC along with any other polysaccharide combinations. However CMCase activity was not found when xylan combined with agarose, alginate and carrageenan were provided as substrate. *Saccharophagus degradans* 2-40 has also been studied for
the induction of various polysaccharase enzymes in the presence of heterologous substrates (Ensor et al, 1999).

6.6 AGARASE ENZYME PURIFICATION:

In this present study agarase enzyme was purified from *Microbulbifer* sp strain CMC-5. The purification was achieved by ultrafiltration, followed by ion exchange and gel filtration chromatography methods. The strain CMC-5 was grown in artificial seawater medium with yeast extract as nitrogen supplement and agarose as carbon substrate. Standardization of various nitrogen sources showed that yeast extract in the presence of agarose produce maximum agarase enzyme activity in the culture supernatant. Hence, artificial seawater based media with 0.2% agarose and 0.2% yeast extract was used. When the culture was grown at RT for 36 h, culture supernatant revealed the presence of one agarase activity band on SDS-Polyacrylamide gel.

Affinity purification of agarase enzymes using cross-linked agarose Sepharose CL-6B has been reported. This single step procedure is simple and elution of bound agarase generally carried out with agar oligosaccharides which bind competitively with agarase enzyme on Sepharose matrix (Groleau and Yaphe 1977; Morrice et al, 1983; Jam et al, 2005). However, when affinity purification using Sepharose CL-6B was attempted during the present study no activity could be detected in the column flow through nor in the eluted fractions (data not shown). Hence, DEAE based ion exchange chromatography was attempted for enzyme purification. 10 kDa ultrafiltration removes small proteins, peptides, amino acids and other low molecular weight products from
the culture supernatant, which are generally by products of the metabolism. This step also helps in buffer exchange and aids in concentrating the culture supernatant from large scale to lab scale for easy handling. In this present study, the culture supernatant was concentrated 10 fold and dialyzed against 20 mM Tris-Cl (pH 7.0) buffer for further chromatographic steps.

Ion exchange chromatography is generally used as first step in the protein purification process as unwanted contaminated proteins can be easily removed. The bound proteins can be eluted easily with buffer containing NaCl gradient. The bound proteins are eluted from the matrix at varying salt concentration and relatively pure protein of interest can be eluted. In this present study, the agarase enzyme was successfully bound to the DEAE-Sepharose matrix and eluted with NaCl gradient. The agarase activity peak was correlated with one of the protein peak. The agarase enzyme was eluted from the column when the concentration of NaCl in the buffer was around 0.2 M NaCl. Majority of bound proteins were eluted at high salt concentration and no agarase activity was observed in these fractions. Agarase enzyme purification in *Bacillus cereus* ASK 202, *Alteromonas* sp. SY37-12, *Agarivorans albus* YKW 34 has been achieved using DEAE Sepharose (Kim et al, 1999; Wang et al, 2006; Fu et al, 2008). Similarly, strong anionic chromatography matrices have also been used for the purification of agarase enzyme (Sugano et al, 1993).

Gel filtration chromatography is often used as final step for the protein purification, where proteins move through the matrix based on their native molecular weight. Purification of agarase enzyme from *Microbulbifer* strain CMC-5 was
achieved after DEAE ion exchange chromatography with Sephacryl S300 HR. The partially purified fraction was fractionated on Sephacryl S300 HR and proteins were identified in the eluted fractions after gel filtration. One of the protein peaks showed agarase activity. Agarase purification in *Pseudoalteromonas* CKT1, *Alteromonas* SY37-12, *Agarivorans albus* YKW 34 and *Vibrio* sp F6 has been achieved using gel filtration (Chiura and Kita-Tsukamoto 2000; Fu et al, 2008a&b).

The agarase enzyme from *Microbulbifer* strain CMC-5 was purified to 103.5 fold with a specific activity of 757.7 U/mg. Only 6.5% recovery was obtained for the purification of agarase enzyme from this strain. Agarase enzyme from *Microbulbifer* JAMB A94 was purified to 220 fold with a specific activity of 517.3 U/mg (Ohta et al, 2004) whereas agarase enzyme from *Vibrio* sp PO 303 was purified to 18.8 fold with specific activity of 16.4 U/mg (Dong et al, 2007). The purified agarase enzyme from *Microbulbifer* strain CMC-5 was referred as agarase enzyme AgaA. The purified agarase enzyme was used for further biochemical characterization.

6.7 BIOCHEMICAL CHARACTERIZATION OF AGARASE ENZYME:

β-agarase enzyme releases neoagarooligosaccharides as degradation products by hydrolyzing β-(1,4) linkages in agar, whereas α-agarase releases agarooligosaccharides by hydrolyzing the α-(1,3) linkage (Potin et al, 1993). Neoagarooligosaccharides have been routinely separated by TLC and detected with 10% H₂SO₄ (Duckworth and Yaphe, 1970; Ohta et al, 2005). After incubation for 24 h, the main products were neoagarohexose and neoagarotetrose as judged by their Rₜ values which
were identical to authentic standards of neoagarohexose and neoagarotetrose used during TLC. Thus the agarase enzyme AgaA from *Microbulbifer* strain CMC-5 is endo-type β-agarase. In the present study the oligosaccharides produced by AgaA hydrolysis of agarose produced neoagaro-oligosaccharides belong to various degree of polymerization. Agarases from several bacteria such as *Vibrio* strain JT0107, *Vibrio* PO-303, *Bacillus* MK03, deep sea *Microbulbifer* strain, *Microbulbifer* JAMB A94, *Pseudoalteromonas* N1 and *Alteromonas* sp SY37-12 have been reported to produce neoagarohexose and neoagarotetrose as by-products during agarose hydrolysis and were detected by TLC (Sugano et al, 1995; Vera et al, 1998; Suzuki et al, 2003; Ohta et al, 2004; Wang et al, 2006; Dong et al, 2007). The agarase enzyme AgaA from *Microbulbifer* strain CMC-5 is not showing any activity, when carrageenan is provided as substrate, which is structurally similar to agarose. The inability to show the activity towards carrageenan showed that the agarase AgaA is agar/agarose specific.

In the present study *in situ* activity of agarase enzyme was detected by SDS-PAGE by incorporating 0.02% LMP grade agarose into the polyacrylamide gel. After the electrophoresis the agarase enzyme activity bands were visualized by lugol’s iodine staining. Ghadi et al, (1997) had reported retardation of agarase in composite polyacrylamide gel. Hosoda et al, (2003) reported that incorporation of low concentrations of agarose in the polyacrylamide gel did not interfere with the migration. In this present study, 0.02% of agarose was incorporated and used for the detection of agarase activity in SDS-polyacrylamide gel. The single activity band
detected by lugol’s iodine staining correlated with the single purified protein band detected by coomassie brilliant blue staining.

The molecular weight of AgaA was determined as ~59 kDa from SDS-PAGE. Agarase enzyme reported from genus Microbulbifer varies from 49 kDa to 126 kDa (Ohta et al, 2004). 16S rDNA of Microbulbifer strain CMC-5 is 99% similarity with M. elongatus DSM 6810. Although M. elongatus DSM 6810T grows on agar, agarase enzyme from this type strain has not been characterized. The agarase AgaA from Microbulbifer strain CMC-5 is closer in molecular weight to agarase of Microbulbifer JAMB A-94, which was reported as ~ 49 kDa agarase enzyme (Ohta et al, 2004). The other agarase enzymes from agarolytic strains molecular weight lies in close vicinity of Microbulbifer strain CMC-5 are Alteromonas strain CKT1 (52 kDa), Pseudoalteromonas sp CY24 (50 kDa), Vibrio sp V134 (50 kDa), Agarivorans albus YKW 34 kDa, Vibrio sp PO 303 (52 kDa) and Vibrio sp. F6 (50 kDa) (Leon et al, 1992; Ma et al, 2007; Zhang et al, 2007; Fu et al, 2008a&b; Dong et al, 2007). Based on molecular weight, agarase enzymes have been classified into three groups. Group I includes agarase with molecular weight of ~30 kDa, Group II includes ~50 kDa whereas Group III includes agarase with ~100 kDa (Vera et al, 1998). The agarase enzymes from Microbulbifer strain CMC-5 belongs to Group II which is characteristic of low molecular weight agarases and are capable of diffusing through gel pores resulting in formation of clearance zone around colonies of Microbulbifer strain CMC-5 during its growth on ASW agar plate. The molecular weight of various agarase enzymes are given in Table 6.2.
AgaA from Microbulbifer strain CMC-5 has an optimum pH of 7.0 and can retain 80% activity at pH 8.0. Majority of the agarase enzyme such as from Pseudomonas atlantica Pseudomonas sp W7, Bacillus cereus, ASK 202 Microbulbifer, Alteromonas SY37-12 Vibrio sp V134 and Vibrio sp F6 have an optimum pH of 7.0 (Morrice et al, 1988; Ha et al, 1997; Kim et al, 1999; Ohta et al, 2004; Wang et al, 2007; Zhang and Sun 2007; Fu et al, 2008a). The optimum temperature of AgaA from strain CMC-5 is 50°C. Agarase enzyme AgaA from Microbulbifer strain CMC-5 retains 74% activity at 60°C. The agarase from closely related Microbulbifer sp strain JAMB-A94 and Microbulbifer strain JAMB-A7 were observed to have optimum temperature of 50 and 55°C respectively (Ohta et al, 2004; Ohta et al, 2005). Further majority of the purified agarase enzymes have an optimum temperature of 40°C. (Kirimura et al, 1999; Suzuki et al, 2003; Zhang et al, 2007; Fu et al, 2008). Few of the agarase enzymes have also been reported with an optimum temperature of 30°C (Morrice et al, 1983; Yamura et al, 1991; Leon et al, 1992; Sugano et al, 1993; Ha et al, 1997; Vera et al, 1998; Kong et al, 2003). It is interesting to note that most of the α-agarase reported so far has optimum temperature of 30°C (Potin et al, 1993; Sugano et al 1994; Suzuki et al, 2002). Recently, agarase enzyme from thermophilic agarolytic bacterium Thermoanaerobacter wiegelli B5 having an optimum temperature of 70°C has been characterized (Bannikova et al, 2008).

The agarase enzyme AgaA from Microbulbifer strain CMC-5 was found to be thermostable at 60°C, whereas it completely lost the activity at 70°C. Agarase enzymes reported form Saccharophagus degradans 2-40 and Microbulbifer bacterial species
also show thermal stability up to 60°C (Whiteland et al., 2001; Ohta et al., 2004). Studies of thermal stability of agarase enzymes from *Microbulbifer* strain CMC-5 indicated that it retains 51% of its activity at 60°C after 30 min of incubation. When compared with its closest relative, agarase from *Microbulbifer* strain JAMB A94 retains 80% of its activity at 65°C for 15 min (Ohta et al., 2004). The agarase enzyme from *Microbulbifer* strain JAMB A7 retains 40% of activity at 80°C after 15 min, whereas agarase from *Microbulbifer* strain CMC-5 loses its activity completely at 70°C (Ohta et al., 2005). Agarase from *Vibrio* sp JT0107 and *Vibrio* V134 were inactive after 20 min of incubation at 60°C and 50°C respectively (Sugano et al., 1997; Zhang and Sun, 2007).

The kinetic studies of agarase enzyme AgaA from *Microbulbifer* strain CMC-5 with agarose as substrate indicated that $K_m$ of agarase enzyme is 1.1 mM whereas $V_{max}$ was found to be 142 U/mg. Similarly agarase enzymes reported from *Zobellia galactinivorans* have $K_m$ of 2 mM in the presence of liquid agarose (Jam et al., 2005). The agarase enzyme from *Alteromonas* strain C1 was reported to has 1.7 mM in the presence of neoagarohexose (Leon et al., 1992) whereas $K_m$ for agarase enzyme from *Vibrio* sp PO 303 was reported as 2.33 mg/ml for agarose (Dong et al., 2007).

<table>
<thead>
<tr>
<th>Name of the Organism</th>
<th>Mol. Wt (KDa)</th>
<th>Optimum pH</th>
<th>Optimum Temp</th>
<th>End products</th>
<th>Reference</th>
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</thead>
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<td>59</td>
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<td>50</td>
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<td>Present study</td>
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<td>Sugano et al., 1993</td>
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NA14: Neoagarotetradecose; NA10: Neoagarodecose; NA8: Neoagarooctose; NA6: Neoagarohexose; NA4: Neoagarotetrose; NA2: Neoagarobiose; G: Galactose; AG5: Agaropentose; AG3: Agarotriose; 3,6-AG: 3,6-anhydrogalactose.

Table 6.2 Salient features of agarase enzymes reported from different microorganisms in comparison to Microbulbifer strain CMC-5.
The EDTA treated and dialyzed enzyme of strain CMC-5 lost 59% of activity indicating an essential requirement of metal ions. Agarase enzymes from *Agarivorans albus* YKW 34 was not affected by EDTA treatment (Fu et al, 2008). Agarase activity from *Vibrio* sp F6 and *Pseudoalteromonas* sp CY24 were inhibited by EDTA treatment whereas the activity was enhanced in *Bacillus* sp MK03 (Suzuki et al, 2003; Ma et al, 2007; Fu et al, 2008a). The dialyzed agarase enzyme was further checked for the activity in the presence of various metal ions and chemical agents. Increase in activity was observed with Ca$^{2+}$, Mg$^{2+}$, CO$^{2+}$ and Mn$^{2+}$, which are commonly observed in marine environment. Agarase enzyme from Microbulbifer strain CMC-5 showed enhanced activity up to 0.5M NaCl. Thus activity of AgaA of *Microbulbifer* was enhanced in the presence of metals and salts which are commonly present in seawater. Na$^{2+}$, K$^{2+}$, Ca$^{2+}$ and Mg$^{2+}$ are known to activate other agarase enzymes (Groleau and Yaphe 1977; Wong et al, 2006, Ha et al, 1997; Lee et al, 2006). However presence of Zn$^{2+}$, Hg$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$ inhibited AgaA from *Microbulbifer* from strain CMC-5 predominantly by binding to SH, CO and NH moieties of amino acids at active site. Similar inhibitory effect has been reported for other agarase (Aoki et al, 1996; Suzuki et al, 2003, Ma et al, 2007; Fu et al, 2008). Agarase enzyme from *Microbulbifer* strain CMC-5 was totally inactivated by β-mercaptoethanol indicating an active role of disulphide bond in protein stability. AgaA from strain CMC-5 was inactivated by N-bromosuccinamid indicating that typtophan play an important role in catalysis (Ohta et al, 2005). Activity of AgaA from *Microbulbifer* strain CMC-5 was observed to increase in presence of 0.1% SDS but was completely inactivated at 1% concentration.
inactivated by SDS (Suzuki et al, 2003). However, agarase from *Agarivorans albus* YKW 34 was observed to be resistant to SDS (Fu et al, 2008).

6.8 ANTIOXIDATIVE PROPERTIES OF OLIGOSACCHARIDES PREPARED BY AGARASE AgaA ENZYME:

Antioxidants can interfere with the oxidation process by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers. In addition, antioxidants effectively retard the onset of lipid oxidation in food products. Oligosaccharides are made of 2-20 sugar units with low degree of polymerization (DP). Increasing awareness of the polysaccharases from marine bacteria has lead to potential applications in various biological fields such as food technology, molecular biology and aquaculture (Weiner et al, 1998; Roberfroid and Slavin 2000; Buddingston 2001).

Reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions and hydrogen peroxide are essentially produced in all aerobic organisms and excess load of these free radicals result in oxidative stress which cause cumulative damage to biomolecules within the cell (Lesser 2006). Protection against oxidative stress can be provided by antioxidants as food additives. Search for non toxic antioxidative substances from natural sources is under extensive study. Marine algae have been recently been exploited as source of antioxidants (Wang et al, 2007). Biologically, antioxidants are defined as synthetic or natural substances added to prevent or delay the deterioration of biological molecules by action of oxygen in air. The antioxidative properties of seaweeds are mainly due to carotenoids and sulphated polysaccharides. These polysaccharides consist mainly of xylose, glucose and galactose units (Geresh
These polysaccharides consist mainly of xylose, glucose and galactose units (Geresh and Arad, 1991). It is believed that the sulphate group attached to the polysaccharides shows strong antioxidative effect over polysaccharides devoid of sulphate group (Wang J et al, 2004). Antioxidative properties of red, brown and green seaweed extracts have been extensively studied (Rocha de Souza et al, 2007; Wang et al, 2007). Agar oligosaccharides prepared by water extraction, methanol extraction, acid hydrolysis and enzyme hydrolysis produce different types of oligosaccharides with different biological activities.

In the present study, AgaA agarase enzyme from Microbulbifer strain CMC-5 was used for application studies. During the present study, water soluble oligosaccharide were prepared from commercial agar by agarase enzyme treatment. The oligosaccharides obtained function as antioxidants showing reducing activity, hydroxyl radical scavenging activity and lipid peroxidation inhibition. Agarase enzyme release agar oligosaccharides from agar in processive manner where in agar is degraded initially into high molecular weight oligosaccharides (with high DP) which on prolonged incubation leads to formation of hexa-, penta- or tetra- oligosaccharides (low DP).

The reducing activities of agar oligosaccharides serve as an indicator of potential anti-oxidant. The reducing activity was mainly due to transformation of Fe$^{3+}$ to Fe$^{2+}$ by oligosaccharides. Oligosaccharides act by preventing chain initiation and binding to transition-metal ion catalysts. In the present study, oligosaccharides showed dose dependent reducing power. Agar oligosaccharides obtained by digestion for 6 h
showed 0.44 µg/ml equivalents of ascorbic acid whereas 12 h oligosaccharides only 0.30 µg/ml equivalents of ascorbic acid. Wang et al, (2004) and Karawita et al, (2005) have also demonstrated dose dependent reducing power of oligosaccharides which were obtained from agar and brown seaweed *Hijikia fusiformis*.

Hydroxy radicals have very short life span but when they are generated, they hydroxylate DNA, proteins and lipids. Transition metals are involved in generation of hydroxy radicals by Fenton reaction \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^* \) (Wang et al, 2007). Hydroxy radicals attack biological molecules in a diffusion controlled manner with a life time of \( 10^{-7} \) seconds and mean diffusion distance 4.5 nm (Lesser 2006). Wang et al, (2004) reported the dose dependent inhibitory effect of hydroxy radical inhibition activity of agar oligosaccharides. The agar oligosaccharides with a degree of polymerization (DP) of 16-24 with one sulphate group on each oligosaccharide showed 76% of hydroxy radical inhibition at 100mg/L in comparison to agar oligosaccharides with low degree of polymerization. Thus agar oligosaccharides play a major role as inhibitors for hydroxy radicals by scavenging them directly. Similarly, alginate oligosaccharides have showed \(~60\%\) of inhibition whereas chitooligosaccharides and fucoidin oligosaccharides have showed \(~30\%\) and \(~20\%\) of hydroxyradical inhibition respectively (Wang et al, 2007).

In present study, agar oligosaccharides prepared from commercial agar by agarase treatment for 6 h, when used at a concentration of 800 mg/L showed 43% inhibition of hydroxy radical formation whereas oligosaccharides obtained by 12 h agar digestion demonstrated 22 % inhibition. Augmentation of hydroxy radical
inhibition by 6 h oligosaccharides is probably due to production of oligosaccharides with high DP in comparison to oligosaccharides with low DP obtained during 12 h digestion.

The reactive oxygen species causes the peroxidation of lipids, by acting on linoleic acid of cellular membranes to form malonaldehyde (MDA), a difunctional aldehyde, which act as cross linking agent for DNA and proteins. It also acts as catalyst for the formation of N-nitrosamines in nitrite containing foods. Therefore, it is very important to reduce the lipid peroxidation in the cells (Eriksson, 1987). Lipid peroxidation of mitochondria can lead to multiple damages to cellular functions and ATP production which further leads to apoptosis (Green and Reed, 1998). Hence, it is very important to reduce the damage caused to the membrane lipids. Agar oligosaccharides obtained from agar during 6 h digest by AgaA, at a concentration of 800 mg/L exhibited 32.6% inhibition of lipid peroxidation whereas oligosaccharides from 12 h digest depicted 26.3% inhibition. Chitin oligosaccharides have been observed to show ~80% of inhibition in lipid peroxidation whereas agar oligosaccharides with a DP of 6-22, without sulphate group, could inhibit ~15% of lipid peroxidation (Wang et al, 2007).

Earlier reports suggested that oligosaccharides from Laminaria japonica, Porphyra haitanesis and Fucus vesiculosus showed antioxidative properties. It is believed that these polysaccharides scavenge the metal ions by chelation (Zhao et al, 2002). Oligosaccharides with different degree of sulphation and high molecular mass (<2000 Da) contribute differently to the antioxidative properties. It is possible that
these agar oligosaccharides may be high molecular mass with high degree of sulphation. However, low molecular mass polysaccharide preparations from Lamina rina japonica have shown antioxidative properties (Xue et al, 2001). Sulphated polysaccharides from Porphyridium sp. have also shown anti viral activity against Herpes simplex virus 1 and 2 and Varicella zoster (Huleihel et al, 2001). Agar oligosaccharides have been reported to perform as moisturizing and whitening agents (Kobayashi et al, 1997). Thus the oligosaccharides obtained by agar digest by agarase AgaA from Microbulbifer strain CMC-5 could be exploited as reducing agent, hydroxyl radical scavenger and inhibitor of lipid peroxidation.

Agar and agarose polysaccharides have highest demand as thickening agents in food industry. They are widely used in gelling, thickening and stabilizing agents for candies and jam. Thus agar has been widely accepted as food additive without any side effects. The agar oligosaccharides which are prepared by hydrolysis of agar by agarase digestion could be added as additives during preparation of jellies, candies and jam which in turn will increase the nutraceutical quality of food in which they are incorporated.

Agarase from Microbulbifer strain CMC-5 showed promising result in production of oligosaccharides which depict reducing power, inhibition of hydroxy radical and lipid peroxidation. In present study, commercial bacterial agar has been used as a source of substrate. Different grades of agar and use of seaweeds as a source for agarase digestion would release oligosaccharides which might have better antioxidative activity due to presence of sulphate groups.
Thus the agarase enzyme from Microbulbifer strain CMC-5 would have potential application in preparation of oligosaccharides from seaweeds and their use as antioxidants as well as possible potential use as moistening and whitening agent which could be analyzed to increase the exploitation of this agarase AgaA enzyme in the future.

6.9 HIGH MOLECULAR WEIGHT DNA RECOVERY FROM AGAROSE GEL:

Recovery of high molecular weight genomic DNA from agarose gel in intact form is essential in various molecular biology applications. A number of methods have been described in literature for isolation of DNA from gel. DNA isolation by freeze thawing of agarose gel, hydroxyapatite chromatography, salt extraction from agarose gel, electrophoretic elution into dialysis bag, continuous elution of DNA from agarose gel by preparative gel electrophoresis, recovery using Sephadex matrix by centrifugation or use of microwave have been reported (Thuring et al, 1975; Tabak and Flavell, 1978; Vogelstein and Gillespie 1979; Dretzen et al, 1981; Fan and Mei, 2005; Chen and Yun 2006; Ouyang et al, 2006). Most of these methods are time consuming or need special equipment and are usually suitable for low molecular weight DNA. Recovery of high molecular weight DNA by any of these method results in poor recovery and shearing. DNA preparations obtained by these methods are also contaminated with traces of agarose. Treatment of agarose gel with agarase enzyme for the recovery of DNA from agarose gel is one of the widely accepted and commercially exploited techniques. Agarase enzyme can easily hydrolyze the agarose and release the DNA from it. The
eluted DNA can be ethanol precipitated for efficient recovery. Normal agarose or low melting agarose are generally used for the analysis of DNA. Agarase enzymes isolated from *Flavobacterium* and *Pseudomonas atlantica* have already been commercially exploited (Tech Bulletin, NEB and Sigma Chemicals). However, many of these commercial agarases have been widely used for low molecular weight recovery and no literature is available regarding recovery of high molecular weight DNA.

In the present study, agarase enzyme AgaA was tested for its ability to recover high molecular weight DNA from agarose gel. *E.coli* genomic DNA was eluted from agarose gel. The agarase AgaA enzyme could efficiently recover the DNA from low melting agarose gel. The recovery efficiency was checked in both TAE and TBE buffer, which are standard buffers for agarose gel electrophoresis. Agarase enzyme AgaA was used as a concentration of 1 U/ml (one unit is the amount of enzyme required to release 1 μM of galactose at 30±2°C per min). Incubation at 50°C for 30 min helps in solubilising the agarose matrix. Since the agarase AgaA has optimum temperature at 50°C, addition of enzyme to the pre heated agarose matrix does not affect AgaA activity. The high molecular weight DNA recovery from agarose gel efficiency is approximately 60% after the treatment with agarase enzyme AgaA. This indicates that AgaA enzyme could efficiently penetrate the agarose matrix and hydrolyze the agarose gel. Complete solubilization of agarose gel is very important for the recovery of nucleic acids. Although chaotropic salts such as NaI or KI are used to reduce the solidification of agarose gels (Vogelstein and Gillespie, 1979), none of them
were used during the present study. No undigested agarose pieces were observed during the present study.

Most of the commercial available agarase enzyme have optimum working condition at pH <7.0. At acidic conditions, agarase enzyme hydrolyzes agarose when it is in complete soluble form. However, in alkaline conditions, they partially hydrolyze the agarose polymer as a result of which DNA molecules remain trapped inside the agarose matrix and leading to reduction in DNA recovery. Agarase enzyme 0107 from *Vibrio* sp. JT0107 which has a pH optimum 8.0 was checked for the recovery of 2.7 Kbp DNA molecules from agarose gel. It was observed the enzyme 0107 could recover 60% of the plasmid DNA, whereas tested commercial agarase could recover only 7% of DNA (Sugano et al, 1993). Fan and Mei (2005) reported 95% recovery of DNA with continuous agarose gel electrophoresis. But these particular methodology required specific operating instruments. Chen and Yun (2006) reported 40-50% recovery of DNA using Sephadex based spin columns. Similarly, 70% of DNA recovery was obtained by freeze-squeeze method and 60-80% recovery by electrophoretic elution into dialysis bag (Thuring et al, 1975; Dretzen et al, 1981). However, recovery of high molecular weight DNA from agarose gel has been always a critical issue to be solved which has not been addressed in any of the above methods.

There are few reports available where agarase enzyme has been used for high molecular weight DNA recovery (Albertsen et al, 1990; Osoegawa et al, 1998). Albertsen et al, (1990) reported the successful recovery of high molecular weight DNA from agarose gels and construction of yeast artificial chromosomes using commercially
available agarase enzyme from Calbiochem Ltd., (Albertsen et al, 1990). However commercially available agarase enzymes have been recommended for recovery of DNA molecules shorter than 500 bp (NEB Technical bulletin).

Although available methods for the recovery of DNA from agarose gels are efficient, they need little more expertise for handling and can perform well with low molecular weight DNA samples such as plasmids or PCR products. Treatment with agarase enzyme is relatively a simple procedure, which involves solubilisation of agarose gel, treatment with agarase enzyme and precipitation with ethanol. Hence, this procedure can be efficiently implemented to recover the high molecular DNA from agarose gels using AgaA enzyme from Microbulbifer strain CMC-5.

6.9 PREPARATION OF ALGAL DETRITUS:
Seaweeds are primary food source for herbivorous fish and they are rich in dietary fibres, minerals, proteins, vitamins and carotenoids. They serve as nutritional sources for aquaculture (Burtin et al, 2003). Algal diets have been extensively used for the cultivation and their effect on yield of aquaculture has been reviewed (Viera et al, 2005). Although seaweeds are rich in nutrients their utilization of seaweed grazing fish species is relatively a difficult task due to high content of indigestible fibre. Both fungi and bacteria play a vital role in utilization of this material from seaweeds since many of them have the ability to digest cellulosic material. In nature, the dead seaweeds tend to settle down at the bottom of the sea bed and these microorganism attacks them to release and utilize the carbon sources. As the degradation of seaweeds by these
easily taken by the surrounding marine animals. Reports suggest that marine animals can easily absorb the nitrogen content available from seaweeds. Detritus prepared with microbial consortia showed ready absorption of nitrogen content in marine animals (Mann et al, 1988). Rice (1982) showed that nitrogen material available during the decomposition of *Spartina* could form condensation products with available amino acids and makes precursors for complex biological processes. Hence provision of seaweed detritus to the artificial aquaculture ponds will certainly enhance the production and yield of commercially important marine organisms.

Microorganisms with decomposing activity of seaweeds have been used in preparation of protoplasmatic detritus from *Laminaria japonica*. Treatments with microbial enzymes render the seaweeds to release cell clumps/single cells. Other nutrients released into the medium during decomposition were also made available to the feeding organisms (Uchida, 1996).

In the present study, agarase enzyme from *Microbulbifer* strain CMC-5 and commercial cellulase have been successfully used to release single cell detritus from seaweeds. The results clearly indicated that the agarase enzyme form *Microbulbifer* strain CMC-5 along with commercially available cellulase enzyme sequentially release algal cell clumps into the surrounding. Such digestion of the seaweed cell wall would increase the bioavailability to the marine organisms. This preliminary study on the preparation of algal cell detritus from *Gracilaria corticata*, showed that agarase enzyme from *Microbulbifer* strain CMC-5 could be efficiently used for the preparation of algal cell detritus, which can be used as feeding material. Single cell detritus can be
used as feed in aquaculture farms especially related to prawn hatcheries. These single cell detritus are natural food and would provide all the essential nutritional requirements for the growth of animals unlike artificial food which could cause proliferation and bacterial contamination leading loss of water quality.

*Microbulbifer* strain CMC-5 is a multiple polysaccharide degrading bacteria which degrades CMC, agar, carrageenan, alginate and other polysaccharides (Section 3.10.4 and 3.13). Also as reported growth of strain CMC-5 results in production of agarase and CMCase (Section 3.13). In the future, enzyme preparation from strain CMC-5 grown in presence of CMC and agar could be obtained and possibly used for preparation of single cell detritus eliminating the addition of commercial cellulases.