Cloning and Characterization of Polysaccharide Degrading Genes from Selected Marine Bacteria

A thesis submitted to Goa University

For the award of degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

Md Imran

Goa University
Taleigao Plateau 403206
Goa, India

November, 2017

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Under the guidance of

Prof. Sanjeev C. Ghadi

Department of Biotechnology

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Certificate

*This is to certify that the thesis entitled "***Cloning and characterization of polysaccharide degrading genes from selected marine bacteria***" submitted by* ***Mr. Md Imran*** *for the Award of the Degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or institution.*

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Place: Goa University
Date:

STATEMENT

I, hereby, state that the present thesis entitled "***Cloning and characterization of polysaccharide degrading genes from selected marine bacteria***" is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place:
Date: Md Imran

Dedicated to………….
My Loving Parents &
My Sweet Wife

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Introduction

Complex polysaccharides (CPs) are composed of repeating units of homo/hetero monosaccharides units linked by various glycosidic linkages and having diverse functional groups. CPs widely present in plants, animals, and microorganisms. They promote structural integrity and shield organisms from predators. In marine ecosystem, seaweeds and crustaceans are the main source of CPs. Additionally the mangrove ecosystem is received a lot of plant litters rich in CPs. Agar, alginate, carrageenan, xylan, pullulan, pectin, cellulose, chitin etc. are the predominant CPs that are widely present in marine organisms. These CPs constitutes a unique source of carbon sink in marine ecosystem and is a crucial source of metabolizable sugars for marine organisms especially those inhabiting nutrient-deficient and extreme environments. The repeating units of monosaccharides are heavily substituted by various functional group rendering CPs recalcitrant and hence they are also referred as Insoluble Complex Polysaccharides (ICPs). Conversion of these ICPs into simpler oligosaccharides/metabolizable sugar require the synergistic action of polysaccharide-degrading enzymes that hydrolyses CPs in to their respective oligosaccharides/metabolizable sugars. Marine bacteria are the major sources of polysaccharide-degrading enzymes. The polysaccharide-degrading enzymes producing bacteria are ubiquitous in marine ecosystem and have been reported from costal water, sediments, deep sea, seaweeds and exoskeleton of crustacean (Li et al. 2011; Jonnadula et al., 2009; Kobayashi et al., 2009; Khambhaty et al., 2007; Ohta and Hatada, 2006; Revathi et al., 2012; Annamalai et al., 2011). *Saccharophagus degradans* 2-40^{T} (isolated from decaying salt marsh cord grass *Spartina alterniflora*) and *Microbulbifer mangrovi* DD-13^{T} (isolated from mangrove ecosystem of Goa, India) are well-known multiple polysaccharide-degrading bacteria that produced maximum number of polysaccharide-degrading extracellular enzymes such as agarase, alginate lyase, carrageenase, chitinase, amylase, xylanase, pullulanse, glucanase etc. that assist

degradation of CPs and contributes to carbon recycling in marine ecosystem (Hutcheson et al. 2011; Vashist et al. 2013). Importantly, theses polysaccharide-degrading enzymes have immense applications in various industries such as paper, pulp, textiles, food, cosmetics etc. Interestingly, the oligosaccharides produced from the polysaccharides by the action of these enzymes have various important therapeutic applications as well. Additionally, use of polysaccharides-degrading enzymes to produce oligosaccharides from the various seaweed polysaccharides is being marketed as nutraceuticals in several countries.

Agar is the main constituent of the cell walls of red algae (Rhodophyceae). It is extracted from *Gelidium*, *Gracilaria* and *Porphyran* spp on industrial scale. Agar is made up of agarose and agaropectin that is composed of of alternating 3-O-linked β-D-galactopyranose (G) and 4-O-linked α-L-galactopyranose (L). The agar is hydrolysed by the agarase. Based on the mode of action or cleavage pattern agarases are classified α-agarase and β-agarase. Although, the α-agarase is rare, β-agarase have been purified from several marine bacteria that have been isolated from different econiches such as sea water, mangrove water, deep sea, seaweeds etc. The agarase enzyme have wide applications in biotechnology.

Pullulan is a water soluble polysaccharides produced by fungus *Aureobasidium pullulans* (Leathers, 2005). It is a polymer of maltotriose units. Pullulan is also known as α-1,4- ;α-1,6-glucan'. The maltotriose consist of three glucose units that are connected by an α-1,4 glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-1,6 glycosidic bond. Pullulan is hydrolysed by enzyme called pullulanase. The pullulanase is also called debranching enzymes because it acts on branch points in pullulan, starch and dextrin. Based on the substrate specificity, pullulanase is classified in to two groups. 1) Type I pullulanase can hydrolyse only α-1,6-glucosidic linkages, and

2) Type II pullulanase can hydrolyse both hydrolyze α-1,6-glucosidic linkages and also α-1,4-glucosidic linkages. The pullulanase has been reported from many mesophilic and thermophilic bacteria (Lee et al., 1997; Hatada et al., 2001; Bertoldo et al., 2004; Gomes et al., 2003). To the best of our knowledge, present study would be the first report of pullulanase from *Microbulbifer* sp.

Alginate is the main structural component of cell wall of brown algae. It consist of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-glucuronate (G) residues and comprises up to 40% of dry weight of seaweeds (Matsubara et al., 2000). Some bacteria are also known to synthesize alginate (Clementi, 1997; Albrecht and Schiller, 2005). Alginate lyase hydrolyses alginate by cleaving the glycosidic bond through a β-elimination reaction mechanism (Gacesa, 1992). Based on the substrate specificity, alginate lyases are categorized as polyM, polyG, and polyMG specific lyases. The alginate lyase producing bacteria have been reported from various marine sources including seaweeds (Li et al., 2011; Jonnadula et al., 2009; Wang et al., 2006; Malissard et al., 1995; Chavagnat et al., 1996), turban shell gut (Fu et al., 2007), sea mud (Wang et al., 2013) and deep sea sediment (Kobayashi et al. 2009).

Carrageenan is a sulfated polysaccharide found in many red seaweeds such as *Kappaphycus alvarezii, Gigartina skottsbergii, Chondrus crispus* and *Eucheuma denticulatum.* The classification of carrageenans depends on the amount and the location of sulfated ester (S) as well as by the presence of 3, 6-anhydro-bridges in the α-linked residues. The κ (kappa), ï (iota) and λ (lambda) carrageenan are distinguished by the presence of one, two or three ester sulfate groups per repeating disaccharide unit respectively and degraded by κ-carrageenase, ï-carrageenase and λ-carrageenase respectively. The carrageenase producing bacteria has been isolated from seaweed (Li et al., 2013; Liu et al., 2011; Sun et al., 2010; Zhou et al., 2008; Mou et al., 2004), sea water

(Vijayaraghavan et al., 2012; Khambhaty et al., 2007) and deep sea (Ohta and Hatada, 2006).

Chitin is the most copious regenerative polymer in the oceans and is a significant source of carbon and nitrogen for marine food web. It is the main component of crustaceans as well as insects exoskeleton. Chitinases are enzymes that degrades chitin. Based on the mode of action, chitinolytic enzymes can be divided into three categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain; endochitinases, which hydrolyze internal β-1, 4- glycoside and β -N-acetylglucosaminidase, which cleaves N-acetyl glucosamine (GlcNAc) units sequentially from the non-reducing end of the substrate (Fukazimo, 1985; Kurita, 2001). The chitinolytic bacteria have been isolated from shrimp/crab shell (Revathi et al., 2012; Annamalai et al., 2011), sea sponge (Han et al., 2009), sea sediment (Annamalai et al., 2010; Guo et al., 2004), sea water (Murao et al., 1991; Hiraga et al., 1997).

In recent years, whole genome sequencing and annotation have been widely used to determine the genomic potential of microbes for polysaccharide degradation. The genome wide screening of polysaccharide-degrading genes enables the holistic identification of polysaccharide-degrading genes. The annotation of genome would help in identifying polysaccharide-degrading genes by sequence based analysis and detection confirmatory conserved domain in the amino acid sequence of the proteins. Based on the amino acid sequence of the polysaccharide-degrading enzymes, the Carbohydrate-Active Enzymes (CAZymes) have classified them into CAZyme families. CAZymes are a group of enzymes that are implicated in the breakdown or modification of glycoconjugates, oligo- and polysaccharides. The polysaccharide-degrading enzymes belongs to the Glycoside hydrolases (GHs) and Polysaccharide lyase family in the CAZy database. In CAZy database, the GHs and PLs are further classified into 145 GH families and 26 PL

families respectively (Lombard et al. 2013). The predominant polysaccharide-degrading enzymes are classified in following CAZy families: agarase (GH16, GH50, GH86), alginate lyase (PL-6, PL7, PL-17), carrageenase (GH82), amylase (GH10, GH13), xylanase (GH10), polygalactouronase (GH28), α-L-arabinofuranosidase (GH29, GH95), α-1,3-L-neoagarooligosaccharide hydrolase (GH117), α-1,3-L-neoagarobiase/neoagarobiose hydrolase (GH117) etc. Additionally, The GHs and PLs are frequently appended with the non-catalytic Carbohydrate Binding Modules (CBMs) that assist the cognate catalytic module by promoting and maintaining the close and prolonged association of enzyme with substrate polysaccharides. The sequence analysis of polysaccharide-degrading genes would enable the classification of enzymes as per the CAZy database and would facilitate the identification of non-catalytic CBMs of respective enzymes. Thus genome sequencing and functional annotation would enable a comprehensive identification of all CAZymes facilitating a holistic understanding of their ecological role and exploiting them for developing novel technologies. Recently, genomes of several polysaccharide-degrading bacteria have been sequenced with emphasis on annotation of CAZyme genes. In present study, the whole genome of *Microbulbifer mangrovi* DD-13^T is sequenced and annotated with emphasis on comprehensive identification of polysaccharide-degrading genes. Furthermore the DD-13^T genome is annotated for the holistic identification CAZymes. In literature, genome sequence of other *Microbulbifer* sp. including *Microbulbifer elongatus* HZ11 (Sun et al., 2014), *Microbulbifer thermotolerans* DAU221 (Lee and choi, 2016), *Microbulbifer agarilyticus* GP101 (Accession no. NZ_CP019650.1) and *Microbulbifer* sp. CCB-MM1 (Accession no. PRJNA305828) are available, however only *Microbulbifer elongatus* HZ11 is annotated with the emphasis on identification of polysaccharide-degrading genes. Previously, the *Saccharophagus degradans* genome has been completely

sequenced, and the CAZyme genes have been elaborately analysed (Weiner et al. 2008). Additionally, the draft genome sequence of polysaccharides-degrading bacteria such as *Flammeovirga* sp. OC4 (Liu et al. 2015), *Bacillus niacin* strain JAM F8 (Kurata et al. 2014), *Formosa agariphila* KMM3901 (Mann et al. 2013), *Alteromonadaceae* sp. strain G7 (Kwak et al. 2012) and *Vibrio* sp. strain EJY3 (Roh et al. 2012) have also been reported. The present study would be the first report of large scale identification of polysaccharide-degrading genes in *Microbulbifer* sp.

Genomic library of an organism is the potent source for screening and obtaining recombinant clones with desired gene that can be used for expression studies. In order to obtain the clone of polysaccharide-degrading genes of selected *Microbulbifer* sp., preparation of genomic library was selected as one of the objective in the present study. Furthermore, cloning of the selected gene in expression vector was undertaken with a purpose to obtain polysaccharide degrading enzyme at higher yield that can be subsequently evaluated for application studies in the field of medical and industrial biotechnology. Cloning and expression of the gene along with the suitable affinity tags would also help in enzyme purification. Therefore, purification of one of the polysaccharide-degrading enzyme and characterization of their biochemical property was selected as the second objective. Additionally, the cloned gene would be re-sequence and characterized using bioinformatics tools. Thus, the third objective of the present study was bioinformatic analysis of the cloned polysaccharide-degrading gene.

1.1 Objective of the research work

Following are the objective of the present study

1. Preparation of genomic library from selected polysaccharide degrading bacteria
2. Purification of polysaccharide degrading enzyme from recombinant clones and biochemical characterization.
3. Sequence analysis of the cloned polysaccharide degrading gene and its comparison with other polysaccharide degrading genes using bioinformatics tools

Review of Literature

Marine environment offers unique and extreme conditions for the inhabiting microbes. In order to cope up with the variable physiochemical conditions observed in marine habitat, microbes produce diverse types of enzyme and secondary metabolites with novel bioactivities. These novel enzymes and compounds enable microbes to survive in fragile environment and incidentally many of these have potential applications in biotechnology and industries. For example, the marine epiphytic bacteria residing on the surface of seaweeds produces diverse polysaccharide-degrading enzymes such as agarase, alginate lyase, carrageenase etc. These polysaccharide-degrading bacteria have been reported from other niches such as coastal and deep sea, marine sediments, mangroves as well as an epiphytes on crustacean species. Most of these marine habitats are nutrient deficient and the metabolizable sugar exclusively exist in the form of complex polysaccharides (CPs) that is not easily metabolized by the inhabiting microbes. Thus polysaccharide-degrading enzymes present in certain microbes degrade CPs enabling carbon recycling. These microbes serve as potential source for isolating polysaccharide-degrading enzymes with unique diverse catalytic and biochemical properties.

The present chapter provides current bibliographic information in relation to the research objectives proposed in the thesis. The chapter also highlights the various polysaccharide-degrading marine bacteria that have been isolated from marine ecosystem, the details on respective polysaccharide-degrading enzymes and their exploitation in the field of biotechnology. Additionally, the chapter also present information on cloning and expression of polysaccharide degrading genes from marine bacteria. Lastly, the chapter also elaborates on genome sequencing of polysaccharide-degrading bacteria and annotation of various carbohydrate active enzymes (CAZymes) involve in polysaccharide degradation.

2.1 Agar-degrading bacteria

Agar degradation is primarily achieved by the group of enzymes referred as agarase. Based on the mode of action, agarases are divided into α- and β-agarases. Thus the agar degradation follows two different pathways i.e. the α-agarase mediated agar degradation pathway and the β-agarase mediated agar degradation pathway. The β-agarase hydrolyze the β-(1,4) glycosidic bonds of agarose to produce neoagarooligosaccharides with β-D-galactopyranose residues at their reducing ends whereas the α-agarases, hydrolyzes the α-(1, 3) glycosidic linkages of neoagarose repetition moieties and produce agaro-oligosaccharides with 3, 6-anhydro α-L-galactose residues at their reducing ends. Growth of agar-degrading bacteria is associated with formation of craters/depression or clearance zone around the bacterial colonies on agar plate (Imran et al., 2017). Furthermore, the agarolytic activity is also determined by adding Lugol's iodine on agar plate and the appearance of clearance zone around agarolytic colonies (Hodgson and Chater 1981). This strategy is frequently employed to detect the agarolytic bacteria.

Agarolytic bacteria producing α-agarase are rare. *Alteromonas agarlyticus* GJ1B and *Thalasomonas* sp. JAMB-A33 are the only bacterial strains reported to produce α-agarase. The former was isolated from sea water whereas the later was isolated from the marine sediment (Potin et al., 1993; Ohta et al., 2005). On the contrary, the β-agarase producing bacteria are ubiquitous in marine environment and have been isolated from various marine sources such as seaweeds, coastal and deep sea, sediments, sea muds etc. The β-agarase producer that were isolated from the seaweeds are *Aquimarina agarilytica* ZC1 (Lin et al., 2017), *Perisobacter* sp. CCB-QB2 (Furusawa et al., 2017), *Flavobacterium* sp. INCH002 (Lavin et al., 2016), *Microbulbifer mangrovi* DD-13 (Vashist et al., 2013), *Pseudomonas* sp. (Gupta et al., 2013), *Alteromonas* sp. GNUM1

(Kim et al., 2012), *Microbulbifer maritimus* (Vijayaraghavan and Rajendran, 2012), *Pseudoalteromonas* sp. AG52 (Oh et al., 2010), *Microbulbifer* strain CMC-5 (Jonnadula et al., 2009), *Alteromonas* sp. SY37-12 (Wang et al., 2006), *Pseudoalteromonas antarctica* N-1 (Vera et al., 1998), *Vibrio* sp. AP-2 (Aoki et al., 1990) and *Pseudomonas atalantica* (Morrice et al., 1983). Likewise, many β-agarase producing bacteria isolated from coastal sea water are *Thalassopira profundimaris* fst-13007 (Zeng et al., 2016), *Pseudoalteromonas* H9 (Chi et al., 2015), *Catenovolum agarivorans* YM01^T (Cui et al., 2014), *Penibacillus* sp. WL (Mei et al., 2014), *Bacillus megatarium* (Khambhaty et al., 2008), *Vibrio* sp. Strain 134 (Zhang and Sun, 2007), *Pseudoalteromonas* sp. CY24 (Ma et al. 2007), *Vibrio* sp. JT0107 (Sugano et al., 1993), *Alteromonas* sp. C-1 (Leon et al., 1992) and *Cytophaga* sp. (Duckworth and Turvey, 1969). Similarly, many researchers also screened agarolytic bacteria from marine sediment. Interestingly, they found β-agarase producing *Pseudoalteromonas* sp. NJ21 (Li et al., 2015), *Flammeovirga* sp. MY04 (Han et al., 2012), *Psychromonas agarivorans* J42-3A^T (Hosoya et al., 2009), *Agaraivorans* sp. HZ105 (Hu et al., 2009) and *Vibrio* sp. P0303 (Araki et al., 1998) from marine sediment. The reports on agarolytic bacteria from deep sea includes the *Flammeovirga* sp. OC4 (Chen et al., 2016) and *Microbulbifer*-like isolate (Ohta et al., 2004). Additionally, the agar-degrading *Vibrio algivorus* (Doi et al., 2016), *Pseudoalteromonas* sp. (Oh et al., 2011) and *Agarivorans albus* YKW-34 (Fu et al., 2008) have been isolated from the gut of marine turban shell whereas *Stenotrophomonas* sp. NTa has been isolated from marine mud (Zhu et al., 2016).

2.1.1 Cloning and expression of agarase gene

The β-agarase gene of several marine bacteria have been cloned and expressed in *E.coli/Bacillus* host system. Many bacteria possess multiple genes for agarase enzymes. For example, three genes of β-agarase namely *agaA, agaD* and *agaC* of *Vibrio* sp. PO-

303 have been cloned and expressed in *E.coli* (Dong et al., 2007a, 2007b, 2006). The *agaA* and *agaC* of *Vibrio* sp. PO-303 have also been expressed in *E.coli* Bl-21 whereas *agaD* was expressed in *E.coli* DH5α (Dong et al., 2007a, 2007b, 2006). Likewise two genes, *agaA* and *agaB* from *Vibrio* sp. JT0107 have been cloned and intracellularly expressed in *E.coli* DH5α. The *Zobellia galactanivorans* also have two genes for β-agarase enzyme. These genes *agaA* and *agaB* have been cloned and expressed in *E.coli* DH5α (Jam et al., 2005). Another multiple polysaccharide-degrading bacterium, *Saccharophagus degradans* 2-40^T harbour five agarase genes and these genes *viz. aga50A, aga16B, aga86C, aga50D* and *aga86D* have been cloned in *E.coli* EP1300 (Ekborg et al., 2006). β-agarase gene has also been cloned from seaweed-degrading bacterium *Saccharophagus* sp. AG21 and expressed in *E.coli* Bl-21 (DE3) (Lee et al., 2013). Additionally, the β-agarase genes from *Agarivorans albus* YKW-34 (Fu et al., 2009), *Agarivorans* sp. LQ48 (Long et al., 2009), *Vibrio* sp. V134 (Zhang et al., 2007), *Pseudoalteromonas* sp. CY24 (Ma et al., 2007), *Agarivorans* sp. JA-1 (Lee et al., 2006), *Pseudomonas* sp. SK38 (Kang et al., 2003) and *Pseudomonas* sp. W7 (Ha et al., 1997) have been cloned and express in *E.coli*. To enhance the expression level, β-agarase genes from many bacteria have been cloned and express in *Bacillus subtilis*. The β-agarase genes from *Microbulbifer*-like JAMB-A94 (Ohta et al., 2004a), *Microbulbifer thermotolerans* JAMB-A94 (Ohta et al., 2004b), *Microbulbifer* sp. JAMB-A7 (Ohta et al., 2004c) have been expressed in *Bacillus subtilis*. Interestingly, the only cloned α-agarase gene of *Thalassomonas* sp. JAMB-A33 has been expressed in *Bacillus subtilis* (Hatada et al., 2006).

2.1.2 CAZY classification of agarase gene/enzyme

Based on the amino acid sequence, the agarase enzyme is classified in to four CAZy families, namely GH16, GH50, GH86 and GH96 (Lombard et al., 2014). The β-

agarase belongs to the GH16, GH50 and GH86 families whereas α-agarases are classified under GH96 in CAZy database. Two agarase of *Vibrio* sp. PO-303 belongs to GH16 family while third one belongs to GH 86. The agarase from *Microbulbifer thermotolerans* JAMB-A94, *Microbulbifer* sp. JAMB-A7, *Pseudomonas* sp. SK38, *Pseudomonas* sp. W7, *Agarivorans albus* YKW-34, *Agarivorans* sp. LQ48, *Zobellia galactanivorans* and *Vibrio* sp. V134 (Zhang and Sun, 2007) were classified under GH16 family. GH50 agarases are reported from *Vibrio* sp. JT0107 (Sugano et al. 1994), *Agarivorans* sp. JA-1 (Lee et al. 2006) and *Agarivorans* sp. JAMB-A11. *Microbulbifer*-like JAMB-A94 encodes GH86 agarase. The *Saccharophagus degradans* 2-40^{T} has complex agarolytic system comprising of five different agarases belonging to GH16, GH50 and GH86 families (Ekborg et al. 2006).

2.1.3 Applications of agarase

The agarase enzyme have wide applications in biotechnology. Agarases have been frequently used for the recovery of DNA from the agarose gel. The agarase from *Vibrio* sp. JT0107 was successfully used for recovering 60% of the loaded DNA from the agarose gel (Sugano et al., 1993). The β-agarase with thermostability up to 60 °C has been used in commercial kit of Takara Company for the extraction of DNA from agarose gel. The potential of agarase to degrade the cell wall of red seaweed have also been employed to obtain various labile substances such as carotenoids, unsaturated fatty acids, vitamins etc. from seaweeds. Additionally, agarases in combination with other polysaccharide-degrading enzymes have been used to isolate protoplast from seaweeds (Araki et al., 1998). Besides their biotechnological applications, agarase enzymes has been explored to produce agar oligosaccharides with novel therapeutic properties. For example, the agar oligosaccharides are reported to exhibit antioxidative properties such as scavenging of hydroxyl free radical and superoxide anion radicals as well as lipid

peroxidation inhibition (Wang et al., 2004; Wu et al., 2005). Further, neoagarooligosaccharides have been reported to inhibit bacterial growth, reduce the rate of starch degradation and improve food quality, when supplemented as low-calorie additive (Giordano et al., 2006)

2.2 Pullulanase producing bacteria

Pullulan is a water soluble polysaccharides produced by fungus *Aureobasidium pullulans* (Leathers, 2005). Pullulan is also known as α-1,4- ;α-1,6-glucan. It is a polymer of maltotriose units. The maltotriose are consist of three glucose units that are connected by an α-1,4 glycosidic bond, whereas the consecutive maltotriose units are connected to each other by an α-1,6 glycosidic bond. Pullulan is hydrolysed by enzyme called pullulanase. The pullulanase is also called debranching enzymes because it acts on branch points in pullulan, starch and dextrin.

The pullulanase has been reported from many mesophilic and thermophilic bacteria (Lee et al., 1997; Hatada et al., 2001; Bertoldo et al., 2004; Gomes et al., 2003). To the best of our knowledge, present study would be the first report of pullulanase from *Microbulbifer* sp.

The pullulanase have been classified into five groups. The classification is based on the substrate specificity of the pullulanase and end product of the enzymatic reaction.

(1) Type I pullulanase (EC 3.2.1.41): The type I pullulanase is also referred as true pullulanases. This group of pullulanases specifically cleaves α-1, 6 glycosidic linkages in pullulan or starch or amylopectin. The end product of type I pullulanase is maltotriose and linear oligosaccharides.

(2) Type II pullulanase: Type II pullulanase frequently designated as amylopullulanase hydrolyse α-1, 6 linkages in pullulan as well as α-1, 4 linkages

of other branch polysaccharides. The end product of the enzymatic reaction is mixture of glucose, maltose, and maltotriose

(3) Pullulan hydrolase type I: also called neopullulanase and hydrolyses α-1, 4 linkages in pullulan. The end product of the enzymatic reaction is panose

(4) Pullulan hydrolase type II (Isopullulanase): This group of pullulanase hydrolyses α-1, 4 linkages in pullulan and produces isopanose as the end product of the enzymatic reaction.

(5) Pullulan hydrolase type III: It can hydrolyses both α-1, 4 and α-1,6 glycosidic linkages in pullulan and produces mixture of maltotriose, panose and maltose as end product.

Mesophilic and thermophilic bacteria are the major source of type-I pullulanase. Pullulanase type-I have been purified from *Thermotoga neapolitana* (Kang et al. 2011), *Thermotoga maritima* (Kriegshauser and Liebl 2000), *Bacillus ceerus* FDTA13 (Nair et al., 2006), *Klebsiella pneumoniae* (Kornacker and Pugsley 1990), *Geobacillus thermoleovorans* (Ayadi et al., 2008), *Fervidobacterium pennivorans* (Bertoldo et al. 1999), *Caldicellulosiruptor saccharolyticus* (Albertson et al. 1997) *Bacillus flavocaldarius* (Suzuki et al. 1991), *Bacillus acidopullulyticus* (Kelly et al. 1994) and *Anaerobranca gottschalkii* (Bertoldo et al. 2004). On the other hand, both aerobic and anaerobic bacteria are reported to produce type-II pullulanase (amylopullulanase), however anaerobic bacteria are the highest producers (Coleman et al., 1987). Many aerobic *Bacillus* spp. and *Geobacillus* spp. are identified as the producer of type-II pullulanase. *Bacillus cereus* H1.5 (Ling et al., 2009), *Bacillus* sp. DSM 405 (Brunswick et al., 1999), *Bacillus* sp. TS-23 (Lin et al., 1996), *Bacillus* sp. KSM-1378 (Ara et al., 1995), *Bacillus sp*. XAL 601 (Lee et al., 1994), *Bacillus* sp. 3183 (Shen et al., 1990), *Bacillus circulans* F-2 (Sata et al., 1989), *Bacillus subtilis* (Takasaki, 1987), *Geobacillus*

thermoleovorans NP33 (Noorwez et al., 2006), *Bacillus* sp. US149 (Roy et al., 2003), and *Geobacillus stearothermophilus* L14 (Zareian et al., 2010) are aerobic bacteria that reportedly produce type-II pullulanase. Many thermophilic anaerobes including *Thermotoga maritime* (Bibel et al., 1998), *Thermococcus profundus* (Kwak et al., 1998), *Thermoanaerobacterium thermosaccharolyticum* (Ganghofner et al., 1998), *Thermoanaerobacter ethanolicus* 39E (Mathupala et al., 1993), *Clostridium thermohydrosulfuricum* Z 21–109 (Saha et al., 1990), *Clostridium thermosulfurogenes* EM1 (Spreinat et al., 1990), *Thermoanaerobium brockii* (Coleman et al., 1987), *Thermoanaerobium* Tok6-B1 (Plant et al., 1987), *Thermoanaerobacter finni* (Koch et al., 1987) and *Clostridium thermohydrosulfuricum* (Hyun et al., 1985) are the producers of type-II pullulanase.

2.2.1 Cloning of pullulanase gene from bacteria

Type I pullulanase have been cloned from several bacteria including *Bacillus megaterium* (Yang et al., 2017), *Paenibacillus barengoltzii* (Liu et al., 2016), *Shewanella arctica* (Elleauche et al., 2015), *Paenibacillus polymyxa* Nws-pp2 (Wei et al., 2015), *Anoxybacillus* sp. LM18-11 (Xu et al., 2014), *Thermus thermophillus* HB27 (Wu et al., 2014), *Exiguobacterium acetylicum* (Qiao et al., 2015), *Bacillus cereus* Nws-bc5 (Wei et al., 2014), *Thermococcus kodakarensis* KODI (Han et al., 2013), *Bacillus* sp. CICIM263 (Li et al., 2012), *Thermotoga neapolitana* (Kang et al., 2011), *Geobacillus thermoleovorans* US105 (Ayadi et al., 2008), *Anaerobranca gottschalkii* (Betoldo et al., 2004), *Bacillus thermoleovorans* US105 (Messaoud et al., 2002), *Fervidobacterium pennavorans* Ven 5 (Bertoldo et al., 1999), *Cardiocellulosiruptor saccharolyticus* (Albertson et al., 1997) and *Bacillus flavocaldarrius* KP1228 (Suzuki et al., 1991).

Type II pullulanase have been cloned from many *Bacillus* sp. and other bacteria such as *Geobacillus thermoleovorans* NSP33 (Nisha and Satyanarayana, 2012), *Lactobacillus plantarum* L137 (Kim et al., 2008, 2009), *Bacillus streothermophilus* TS-23 (Chen et al., 2001), *Bacillus* sp. KSM-1378 (Hatada et al., 1996), *Bacillus* sp. strain XAL601 (Lee et al., 1994), *Thermoanaerobacter saccharolyticum* B6A-R1 (Ramesh et al., 1994), *Thermoanaerobacter ethanolicus* 39E (Mathupala et al.,1993; Lin and Liu, 2002), *Clostridium thermohydrosulfuricum* DSM3783 (Melasniemi and Paloheimo, 1989), and *Thermoanaerobium brockii* (Coleman et al., 1987). Additionally, the amylopullulanase have been cloned from a deep sea bacterium *Thermococcus siculi* (Jiao et al., 2011). The type II pullulanase of *Thermococcus Kodakarensis* KODI (Gaun et al., 2013), *Thermococcus hydrothermalis* (Erra-Pujada et al., 1999) and *Pyrococcus furiosus* (Dong et al., 1997) have been also cloned and characterized.

2.2.2 Biochemical properties of native pullulanase

The pullulanase enzyme is a high molecular weight protein. The molecular weight of pullulanase purified from *Thermoanaerobacter* strain B6A is 450 KDa (Saha et al. 1989). Likewise, the molecular weight of type II pullulanase purified from *Bacillus circulans* F-2, *Bacillus* sp. KSM-1378, *Thermoanobacterium thermosaccharolyticum*, *Thermoanobacter ethanolicus* 39E and *Bacillus* sp. DSM 405 are 220 KDa, 210 KDa, 150 KDa, 133 KDa and 126 KDa respectively (Sata et al., 1989; Ara et al., 1995; Ganghofner et al., 1998; Mathupala and Zeikus, 1993; Brunswick et al., 1999). However, relatively low molecular weight pullulanase are also reported from *Geobacillus stearothermophilus* L14 (100 KDa) and *Lactobacillus amylophilus* GV6 (90 KDa) (Zarein et al., 2010; Vishnu et al., 2006)

The pullulanase shows optimum activity in the temperature range of 37 – 65℃. The pullulanase purified from *Lactobacillus amylophilus* GV6 demonstrated optimum activity at 37℃ (Vishnu et al., 2006) whereas the pullulanase from *Bacillus* sp. KSM-1378 and *Bacillus circulans* F-2 demonstrated optimum activity at 50℃ (Ara et al., 1995; Sata et al., 1989). Furthermore, the pullulanase obtained from *Thermoanaerobacter ethanolicus* 39E is optimally active at 60℃ (Mathupala and Zeikus, 1993). The type II pullulanase isolated from *Thermoanaerobacter* strain B6A, *Thermoanaerobacterium thermosaccharolyticum* DSM 571 and *Geobacillus stearothermophilus* L14 (Zareian et al., 2010; Ganghofner et al., 1998; Saha et al., 1990) depicted optimal activity at 65℃ whereas the pullulanase from *Bacillus* sp. DSM405 demonstrated maximum activity at 70℃ (Brunswick et al., 1999).

Type II pullulanase purified from various bacteria showed an optimum activity at wide range of pH i.e. 5- 9.5. The pullulanase from *Bacillus* sp. KSM-1378 demonstrate maximum activity at pH 9.5 (Ara et al., 1995) whereas pullulanase from *Bacillus circulans* F-2 showed optimum activity at neutral pH (Sata et al., 1989). The pullulanase from *Bacillus* sp. DSM 405 and *Lactobacillus amylophilus* GV6 showed optimal activity at slightly acidic pH i.e. pH 6.0 and 6.5 respectively (Brunswick et al., 1999; Vishnu et al., 2006). The pullulanase active at moderate acidic pH are reported from *Theroanobacter ethanolicus* 39E, *Thermoanobacterium thermosaccharolyticum* DSM475, *Geobacillus stearothermophilus* L14 and *Thermoanaerobacter* strain B6A (Mathupala and Zeikus, 1993; Ganghofner et al., 1998; Zarein et al., 2010; Saha et al., 1990).

2.2.3 Biochemical properties of recombinant pullulanase

The pullulanase expressed in homologous/heterologous host have been purified and characterized by several researchers. The molecular weight of recombinant pullulanase from *Bacillus* sp. KSM-1378 is 210 KDa whereas that from *Bacillus* sp. strain XAL601 is 225 KDa (Hatada et al., 1996; Lee et al., 1994). Furthermore, the molecular weight of recombinant pullulanase from *lactobacillus plantarum* L137, *thermoanaerobacterium saccharolyticum* B6A-R1 and *Geobacillus thermoleovorans* NP33 are 215.6 KDa, 142 KDa and 182 KDa respectively (Kim et al., 2008; Ramesh et al., 1994; Nisha and Satyanarayana, 2012). Likewise, the molecular weight of thermostable region of recombinant pullulanase from *thermoanaerobacter ethanolicus* 39E is 109 KDa (Mathupala et al., 1993; Lin and Leu, 2002).

Predominantly, recombinant pullulanase shows optimal activity at higher temperature. The recombinant pullulanase of *thermoanaerobacter ethanolicus* 39E demonstrated maximum activity at 90℃ whereas the pullulanase from *Bacillus* sp. strain XAL601 is optimally active at 70 ℃ (Lin and Leu et al., 2002; Lee et al., 1994). Furthermore, the recombinant pullulanase from *Thermoanaerobacterium saccharolyticum* B6A-R1 and *Geobacillus thermoleovorans* NP33 depicted maximum activity at 65℃ and 60℃ respectively (Ramesh et al., 1994; Nisha and Satyanarayana, 2012). The recombinant pullulanase from *lactobacillus plantarum* L137 showed maximum activity at relatively lower temperature i.e. 40- 45℃ (Kim et al., 2008; 2009).

Recombinant pullulanase of bacteria shows optimum activity at wide range of pH i.e. 4- 9.5. The recombinant pullulanase of *Bacillus* sp. KSM-1378 and *Bacillus* sp. strain XAL601 showed optimum activity at pH 9.5 and 9.0 respectively (Hatada et al., 1996; Lee at al., 1994). The recombinant pullulanase showing optimum activity at neutral pH

is reported from *Geobacillus thermoleovorans* NP33 (Nisha and Satyanarayana, 2012). Furthermore, recombinant pullulanase from *Thermoanaerobacter ethanolicus* 39E and *Thermoanaerobacterium saccharolyticum* B6A-R1 demonstrate optimum activity at slight acidic pH i.e. 6.0 (Lin and Leu, 2002; Ramesh et al., 1994). The pullulanase cloned from *Lactobacillus plantarum* L137 also showed optimum activity at pH 4.0 – 5.0 (Kim et al., 2008; 2009).

2.2.4 CAZy classification of pullulanase

Pullulanase have been classified under GH-13 and GH-57 family of CAZy database (Lombard et al., 2010). The pullulanase from *Lactobacillus amylophilus* GV6, *Bacillus* sp. KSM-1378, *Bacillus circulans* F-2, *Thermoanaerobacter ethanolicus* 39E, *Thermoanaerobacter* strain B6A, *Thermoanaerobacterium thermosaccharolyticum* DSM 571, *Geobacillus stearothermophilus* L14 and *Bacillus* sp. DSM405 belongs to GH-13 family (Vishnu et al., 2006; Ara et al., 1995; Sata et al., 1989; Mathupala and Zeikus, 1993; Zareian et al., 2010; Ganghofner et al., 1998; Saha et al., 1990; Brunswick et al., 1999). GH57 pullulanase have been reported from *Thermococcus siculi* (Jiao et al., 2011), *Thermococcus hydrothermalis* (Erra-Pujada et al., 1999), *Pyrococcus furiosus* (Dong et al., 1997), *Pyrococcus woesei* (Rudiger et al., 1995) and *Thermococcus litoralis* (Brown et al., 1993)

2.2.5 Applications of pullulanase

Like other polysaccharide-degrading enzymes, pullulanase also have wide range of industrial applications. Pullulanase are used for the saccharification of starch to obtained high content glucose, fructose and maltose syrups (Van der Maarel et al., 2002; Gomes et al., 2003). Pullulanase have been exploited to obtained high-amylose starches, that has high market demand as it can be processed to the resistant-starch of nutritional

benefits (Vorwerg et al., 2002). Additionally, the pullulanase is employed to produce cyclodextrins that is used as complexing materials in foods, pharmaceuticals, plastics, emulsifiers, antioxidants, and as stabilizer (Rendleman Jr, 1997; Kim et al., 2000). Other applications include manufacturing of low-calorie beer (Olsen et al., 2000), the antistaling agent in bakery industry (Van der Maarel et al. 2002) and as a dental plaque control agent (Marotta et al., 2002).

2.3 Carrageenase producing bacteria

Like agarolytic bacteria, carrageenase producing bacteria are also ubiquitous in marine environment and are widely isolated from seaweeds. The κ-carrageenase producing *Zobellia* sp. ZM2 (Liu et al., 2013), *Cytophaga drobachiensis* (Barbeyron et al., 1998), *Cytophaga*-like bacterium (Potin et al., 1991), *Tamlana* sp. HC4 (Sun et al., 2010), *Vibrio* sp. CA-1004 (Araki et al., 1999), *Cytophaga* MCA-2 (Mou et al., 2004), *Pseudoalteromonas*-like bacterium WZUC10 (Zhou et al., 2008), *Pseudoalteromonas* sp. QY203 (Li et al., 2013), *Zobellia galactanivorans* (Potin et al., 1991), *Cytophaga* sp. 1K-C783 (Sarwar et al., 1987), have been isolated from seaweeds. Likewise ï-carrageenase producing *Cellulophaga* sp. QY3 (Ma et al., 2013), *Zobellia galactanovorans* (Barbeyron et al., 2000) and *Pseudoalteromonas porphyrae* (Liu et al., 2011) have been also reportedly isolated from seaweed samples. Many κ-carrageenase producing bacteria viz. *Bacillus subtilis* (Vijayaraghavan et al., 2012), *Pseudomonas carrageenovora* (Mclean and Williamson, 1979) and *Pseudomonas elongata* (Khambhaty et al., 2007) have been isolated from sea water. Deep sea sediment have been also screened for the carrageenase producing bacteria and interestingly, κ-carrageenase producing *Pseudoalteromonas tetrodonis* has been isolated from deep sea sediment (Kobayashi et al., 2012). A λ-carrageenase producing *Pseudoalteromonas* sp. strain CL19 has been also reported from deep sea (Ohta and Hatada, 2006).

2.3.1 Cloning of carrageenase gene from bacteria

Carrageenase have been predominantly reported from several marine bacteria and many of them have been cloned and expressed in heterologous host such as *E.coli*. However *Bacillus subtilis* has been also used as alternative heterologous host for the expression of carrageenase gene. The carrageenase gene of *Alteromonas carrageenovora* ATCC 43555 (Barbeyron et al., 1994), *Alteromonas fortis* (Michel et al., 2000; Michel et al., 2001a, b), *Cellulophaga* sp. QY3 (Ma et al., 2013), *Cytophaga drobachiensis* (Barbeyron et al., 1998), *Pseudoalteromonas carrageenovora* ATCC- 43555 (Guibet et al., 2007), *Pseudoalteromonas carrageenovora* (Michel et al., 1999), *Pseudoalteromonas tetraodonis* JAM-K142 (Kobayashi et al., 2012), *Zobellia* sp. ZM-2 (Liu et al., 2013) and *Zobellia galactanivorans* (Barbeyron et al., 2000) have been cloned and expressed in *E.coli*. Furthermore, the carrageenase gene of *Microbulbifer thermotolerans* JAMBA94 has been expressed in *Bacillus subtilis* (Hatada et al., 2011).

2.3.3 CAZy classification of carrageenase

In CAZy database the ï-carrageenase and κ-carrageenase are classified under GH-82 and GH-16 family respectively (Lombard et al., 2010). The ï-carrageenase belonging to GH-82 family have been reported from *Alteromonas* sp. ATCC 43554, *Cellulophaga* sp. QY3, *Microbulbifer thermotolerans* JAMB-A94, *Zobellia galactanivorans* DsiJT (Barbeyron et al., 2000; Ma et al., 2013a; Ma et al., 2013b; Hatada et al., 2011; Thomas et al., 2012). Furthermore, the κ-carrageenase of *Pseudoalteromonas carrageenovora* ATCC 43555, *Pseudoalteromonas porphyrae* LL1, *Pseudoalteromonas tetraodonis* JAM-K142, *Zobellia galactanivorans DsiJT* and *Zobellia* sp. M-2 belonged to GH-16 family (Barbeyron et al., 1994; Liu et al., 2011; Kobayashi et al., 2012; Thomas et al., 2012; Liu et al., 2013).

2.3.4 Application of carrageenase

The carrageenase have also drawn considerable interest. Sulfated oligosaccharides produced by carrageenase enzyme have demonstrated diverse biological and physiological activities, such as anticoagulation (Alban et al., 2002), anti-inflammation (Arfros and Ley, 1993), anti-thrombosis (Suzuki et al., 1991), antitumor activity (Hiroishi et al., 2001) and viral inactivation (Caceres et al., 2000).

2.4 Alginate lyase producing bacteria

The alginate lyase have been purified from various marine bacteria isolated from different sources including marine algae, sea mud, deep sea sediment etc. *Alginovibrio aquatilis* (Steven and Levin, 1977), *Alteromonas* sp. strain H-4 (Sawabe et al., 1992), *Beneckea pelagia* (Sutherland and Keen, 1981), *Halomonas marina* (Kraiwattanapong et al., 1999), *Photobacterium* sp. (Malissard et al., 1995), *Pseudomonas* sp. (Muramatsu and Sogi, 1990), *Pseudomonas alginovora* strain X017 (Boyen et al., 1990), *Vibrio* sp. (Chavagnat et al., 1996), *Vibrio alginolyticus* (Kitamikado et al., 1992), *Vibrio harveyi* AL-128 (Kitamikado et al., 1990), *Vibrio* sp. YWA (Wang et al., 2006), *Pseudoalteromonas* sp. SM0524 (Li et al., 2011) have been isolated from various marine algae. Additionally, the alginate lyase producing *Vibrio* sp. YKW-34 (Fu et al., 2007), *Vibrio* sp. QY105 (Wang et al., 2013) and *Agarivorans* sp. JAM-A1m (Kobayashi et al., 2009) have been isolated from turban shell gut, sea mud and deep sea sediment respectively.

Based on the mode of action, alginate lyase are classified into two groups: the exo- and endo-type alginate lyases. The endo-type alginate lyase is classified as poly-G, poly-M and poly-MG specific alginate lyase. The poly-G and poly-M specific alginate lyase degrade poly-M and poly-G block of alginate respectively whereas the poly-MG

can cleaves both poly-M and poly-G blocks in alginate. The endo-type alginate lyase produces di-, tri- and tetra-saccharides as end products of the enzymatic reactions (Zhang et al., 2004). On the other hand, the exo-type alginate lyase hydrolyses both oligomeric alginates and alginate (Hashimoto et al., 1998). *Sphingomonas* sp. A1 produces oligoalginate lyase that recognizes and degrade both saturated and unsaturated saccharides in alginate/oligoalginate to produce saturated/unsaturated monosaccharides (Miyake et al., 2003).

The alginate lyase purified from *Halomonas marina* (Kraiwattanapong et al., 1999), *Pseudomonas alginovora* strain X017 (Boyen et al., 1990), *Vibrio* sp. (Chavagnat et al., 1996) and *Vibrio* sp. YWA (Wang et al., 2006) have been identified as poly-M alginate lyase whereas alginate lyase of *Pseudomonas* sp. (Muramatsu and Sogi, 1990), *Vibrio alginolyticus* (Kitamikado et al., 1992), *Pseudoalteromonas* sp. SM0524 (Li et al., 2011) are classified as poly-G alginate lyase. Similarly, *Alteromonas* sp. strain H-4 (Sawabe et al., 1992), *Photobacterium* sp. (Malissard et al., 1995), *Vibrio* sp. YKW-34 (Fu et al., 2007), *Vibrio* sp. QY105 (Wang et al., 2013) and *Agarivorans* sp. JAM-A1m (Kobayashi et al., 2009) are the producers of poly-MG alginate lyase.

2.4.1 Cloning of alginate lyase gene

Alginate lyase is produced by several marine bacteria and terrestrial *Pseudomonas aeruginosa*. Gene encoding alginate lyase have been cloned and characterize from several marine bacteria including *Agaraivorans* sp. L11 (Li et al., 2015), *Pseudoalteromonas* sp. CY24 (Duan et al., 2009), *Microbulbifer* sp. 6532A (Swift et al., 2014), *Vibrio* sp. QD-5 (Chao et al., 2017), *Shewanella* sp. (Wang et al., 2015), *Streptomyces* sp. ALG-5 (Kim et al., 2009), *Vibrio* sp. SY08 (Li et al., 2016) and *Pseudomonas* sp. OS-ALG-9 (Kraiwattanapong et al., 1997). Furthermore, three genes

namely A9mT, A9mC and A9mL of deep sea *Vibrio* sp. JAM-A9m encoding alginate lyases have been cloned and characterized (Uchimura et al., 2010). Additionally, the alginate lyase encoding gene, A1mU, from deep sea *Agarivorans* sp. JAM-A1m is also cloned and characterized (Uchimura et al., 2010). The oligo-alginate lyase gene from *Sphingomonas* sp. MJ-3 has also been cloned and expressed in heterologous host (Park et al., 2012). Furthermore, the alginate lyase gene from terrestrial *Pseudomonas aeruginosa* has also been cloned and characterized (Schiller et al., 1993).

2.4.4 CAZy classification of alginate lyase

The alginate lyase/oligo-alginate lyases are classified under PL family in CAZy database (Lombard et al., 2013). In CAZy database, the PL family is further classified in 27 sub-families based on the amino acid sequence similarities intended to reflect the structural features of the member enzymes (Lombard et al. 2013). Till date, alginate lyase/oligo-alginate lyase belonging to PL-5, PL-6, PL-7, PL-14, PL-15, PL-17 and PL-18 have been reported. Following are the reported alginate lyase and their respective PL family: *Sphingomonas* sp. A1 (PL-15), *Flavobacterium* sp. S20 (PL-7), *Pseudomonas* sp. E03 (PL-5), *Saccharophagus degradans* 2-40^{T} (PL-17), *Pseudoalteromonas* sp. SM0524 (PL-18) and *Shewanella* sp. Kz7 (PL-6). (Hashimoto et al., 2005; Huang et al., 2013; Zhu et al., 2015; Park et al., 2014; Dong et al., 2014; Li et al., 2015)

2.4.5 Applications of alginate lyase

Similarly like agarase, alginate lyase have immense medical and biotechnological applications. The alginate oligosaccharides have been reported to stimulate secretion of cytotoxic cytokines by human macrophages (Iwamoto et al., 2005), activate vascular endothelial growth factor mediated growth, migration of human endothelial cells (Kawada et al., 1999), increased tumor necrosis factor-α (TNF-α) secretion from the

macrophage cell line RAW264.7 (Kurachi et al., 2005) and stall the production of reactive oxygen species in immune cells (Courtois, 2009). Alginate lyase have also been used in medical application. Pathogenic bacteria infecting lungs secrete exopolysaccharides that promotes formation of bacterial biofilm and is important for the virulence. The alginate synthesized by *P. aeruginosa* is resistant to degradation as the C2 and/or C3 positions on the some of the M residues are O-acetylated. This makes P. aeruginosa cells resistant to phagocytic cells and/or antibiotics (Cotton et al., 2009). The co-administration of alginate lyase to patients degrades the extracellular alginate biofilm produced by pathogen and increases the efficacy of antibiotic action on the pathogens trapped in the mucus. Thus alginate lyase has the potential to be used as biopharmaceuticals for treatment of bacterial mucoid biofilm dependent diseases.

2.5 Chitinase producing bacteria

Chitin-degrading bacteria are widely distributed in marine environment and are reported from marine sediment, sea sponge, shrimp/crabs shell etc. The *Micrococcus* sp. AG84 and *Aeromonas schubertii* are the chitinase producing bacteria isolated from the marine sediment (Annamalai et al., 2010; Guo et al., 2004). *Streptomyces* sp. DA11 (Han et al., 2009) has been isolated from sea sponge whereas *Vibrio* sp. (Revathi et al., 2012) and *Alkaligen faecalis* AU02 (Annamalai et al., 2011) were reported from shrimp/crab shell. The chitinolytic *Vibrio alginolyticus* TK-22 was isolated from sea water (Murao et al. 1991). The chitinase producing bacteria also includes *Vibrio* sp. 98CJ11027 (Park et al., 2000), *Streptomyces* sp. M-20 (Kim et al., 2003), *Aeromonas hydrophila* H-2330 (Hiraga et al., 1997), *Enterobacter* sp. (Dahiya et al., 2005), *Pseudomonas aeruginosa* (Wang and Chang, 1997), *Vibrio alginolyticus*-H-8 (Ohishi et al., 1996) and *Arthrobacter* sp.NHB-10 (Okazaki et al., 1999).

2.5.1 Cloning of chitinase gene from bacteria

Chitinase encoding genes from several marine and terrestrial bacteria including *Serratia marcescens* (Jones et al., 1986), *Bacillus circulans* (Watanabe et al., 1992), *Vibrio harveyi* (Soto-Gil, 1984), *Vibrio vulnificus* (Wortman et al., 1986), *Alteromonas* sp. strain O-7 (Tsujibo et al., 1993), *Glaciozyme Antarctica* PI12 (Ramli et al., 2011), *Isaria fumosorosea* (Meng et al., 2015), *Streptomyces lividans* 66 (Miyashita et al., 1991) and *Microbulbifer degradans* 2-40 (Howard et al., 2004) have been cloned and characterized.

2.5.2 CAZy classification of chitinase

Chitinase is classified under GH18 and GH19 families of CAZy database (Lombard et al., 2010).

2.5.3 Applications of chitinase

Chitinases have been employed in human health care for developing ophthalmic preparations with chitinases and microbiocides (Dahiya et al., 2006). Chitinases have been also used in the therapy against fungal diseases in potentiating the activity of antifungal drugs (Pope and Davis 1979; Orunsi and Trinci, 1985)

2.6 Applications of other polysaccharide-degrading enzymes

Other polysaccharide-degrading enzymes like xylanases, pectinases, cellulases has wide industrial, biotechnological and pharmaceutical applications. The sources, properties and applications of xylanses have been reviewed by Chakdar et al., (2016). The xylanase has been extensively used in paper and pulp industry, deinking of waste paper, in preparation of animal feeds, in bakery industry and biofuel production (Chakdar et al., 2016). Similarly, the pectinase enzyme is also a commercially important enzyme

as it has various industrial applications. Pectinases has been used to improve the quality and yield of fruit juice, maceration of tea leaves (Angayarkanni et al., 2002), cotton fabric processing (Solbak et al., 2005). The cellulase has drawn the considerable attention for obtaining the second generation biofuel from cellulosic feedstock.

2.7 Genome wide detection of polysaccharide-degrading genes and their classification in CAZy families

Recently genome sequencing and annotation has revolutionized the identification of polysaccharide-degrading genes. Additionally, the sequence analysis of the polysaccharide-degrading genes facilitate their classification to appropriate CAZy families. Earlier genome analysis indicates, far reaching capability of marine bacteria to degrade diverse complex polysaccharides. *Saccharophagus degradans* 2-40^T genome encodes 128 GHs that catabolizes cellulose, substituted xylans, xyloglucans, arabinans and arabinogalactans, pectin and rhamnogalcturonan; β-1,3(4) glucan, β-1,3-glucans, starch, glycogen, pullulan; mannans, glucomannans and galactomannans. In addition to GHs, *Saccharophagus degradans* also encodes 127 identifiable CBMs. Out of 128 GHs, 43 are appended to at least one CBM (Weiner et al. 2008). Similarly, genome sequencing and CAZyme analysis of *Formosa agariphila* KMM 3901 reveals broad potential of the strain to degrade algal polysaccharides (Mann et al. 2013). *Formosa agariphila* KMM 3901 encodes 193 CAZymes that includes 88 GHs, 70 GTs, 13 PLs, 13 CEs, and 9 CBMs. The GHs and PLs of *Formosa agariphila* KMM 3901 distributed in 27 and 4 known families respectively are indicative of the degradation of agar/agarose, alginate, arabinan, fucosides/fucoidan, α-glucans (starch), laminarin, mannan, polygalacturonans, porphyran, and xylan. Likewise, genome annotation of *Microbulbifer elongatus* HZ11 reports several genes concern with degradation of brown seaweed and other polysaccharide (Sun et al. 2014). Strain HZ11 encodes 9 alginate lyase belonging to four

different PL families such as PL6, PL7, PL17 and PL18. Strain HZ11 also encodes 5 agarases belonging to GH16, GH50 and GH86 family. In addition to alginate lyase and agarase, strain HZ11 encodes one cellulase and two amylases. Furthermore, recently sequenced *Algibacter alginolytica* HZ22 has 255 CAZymes including 104 GHs, 18 PLs, 99 GTs, 29 CEs and 13 CBMs (Sun et al. 2016). The GHs of strain HZ22 belong to 29 different families while PLs are classified in to 7 different families such as PL1, PL6, PL7, PL8, PL10, PL12 and PL17. Thus GHs and PLs from the bacterial strain HZ22 demonstrates the potential to degrade various algal polysaccharides such as agarose, fucoidan, fucoside (N-linked glycan), homogalacturonan, rhamnogalacturonan, starch and xylan (Sun et al. 2016). In the draft genome sequence from *Paraglaciecola* sp. strain S66, 288 CAZymes were identified including 113 GHs, 17 PLs, 58 CEs, 35 GTs, 46 CBMs and 11 proteins with auxiliary activities (AAs) (Schultz-Johansen et al. 2016). Further annotation of predicted CAZymes suggests that *Paraglaciecola* sp. S66 can degrade and utilize a range of complex polysaccharides found in algal cell walls that includes, agar (GH16, GH50, and GH86), alginate (PL6, PL7, PL14, and PL17), pectin (GH28, GH88, GH105, PL1, PL9, PL10, and PL11), carrageenans (GH16 and GH82), and xylan (GH10 and GH11) (Schultz-Johansen et al. 2016). The genome of agarose, alginate, starch, and laminarin degrading *Bacillus weihaiensis* Alg07 encodes 32 GHs, 31 GTs, 3 PLs, 29 CEs and 23 CBMs (Zhu et al. 2016). Out of three the PLs from strain Alg07, 2 were annotated as oligo alginate lyase and belonged to PL15 family. The other PL is annotated as alginate lyase of PL17 family. The major GHs of strain Alg07 are β –amylase (GH14), pullulanase (GH13), β –glucosidase (GH1) endo-β -1,3–1,4 glucanase (GH16) and β glucanase (GH16) (Zhu et al. 2016).

2.8 Modular nature of polysaccharide-degrading enzymes

Interestingly, genome sequence analysis and CAZyme prediction reveals the modular nature of various polysaccharide-degrading enzymes. The Glycoside hydrolases and Polysaccharide lyases are appended with CBMs and other non-catalytic modules. The enzymes which contain more than one modules are called modular enzymes. CAZymes modularity are extra advantageous for polysaccharide degradation as the non-catalytic modules increase the degradative potential of their cognate catalytic modules by increasing the substrate concentration on the surface of enzymes. The role of these cognate non-catalytic modules are crucial in natural marine ecosystems where the enzyme concentration becomes diluted due to the water current. In literature, many CAZymes of modular nature are reported. *Saccharophagus degradans* encodes large number of CAZymes with many novel combination of non-catalytic CBMs (Weiner et al. 2008). GH86 β– agarase II (AgaE Sde-2655) of *Saccharophagus degradans* is appended with three CBM6 modules. Additionally endoglucanase 5A of *Saccharophagus degradans* 2-40 have two catalytic GH5 modules and appended with three CBM6 modules (Fig 2.1). *Saccharophagus degradans* also encodes highly modular putative manan endo 1,4-B-mannosidase, putative bifunctional xylanase/ α-L-arabinofuranosidase, Pectin/Pectate lyase, alginate lyase and bifunctional xylanase/ acetylxylan esterase (Fig 2.1). The modular α-agarase and alginate lyase are also reported from *Alteromonas agarilytica* and *Flammeovirga* sp. MY04 respectively (Fig 2.1).

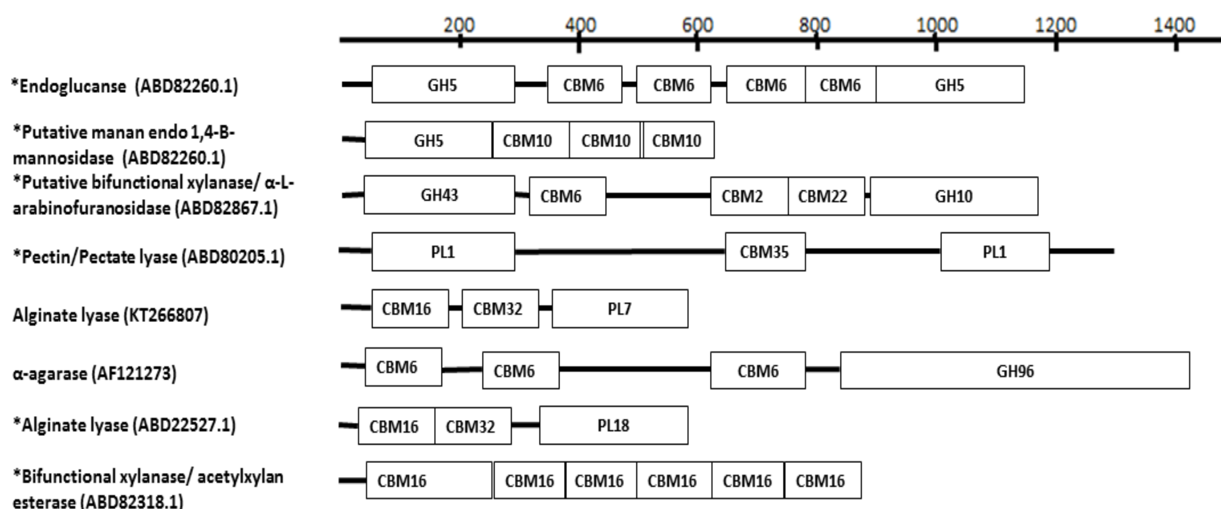


Fig. 2.1: Examples of modular Glycoside hydrolase (GHs) and Polysaccharide lyase (PLs). The scale on the top depicts the amino acid sequence position. *Proteins are from *Saccharophagus degradans* 2-40. The α-agarase and alginate lyase are from *Alteromonas agarilytica* and *Flammeovirga* sp. MY04 respectively.

(Note: the accession numbers of the proteins obtained from the CAZy database/reference article, and analysed using dbCan (http://csbl.bmb.uga.edu/dbCAN/annotate.php). Based on the results of dbcan the approximate position of individual domains represented).

2.9 CAZy classification unravel mechanism of polysaccharide degradation

The polysaccharide-degrading enzymes belonging to Glycoside hydrolases (GHs) and Polysaccharide lyase (PLs) follows the different mechanism for the degradation of their respective substrate polysaccharides. GHs hydrolyse the glycosidic bond either via overall retention or via overall inversion of the anomeric configuration (Davies and Henrissat, 1995). Frequently, hydrolysis of glycosidic bond is attributed to two amino acid residues of the enzyme designated as catalytic residues. One catalytic amino acid acts as proton donor (acid) while another acts as nucleophile (base). Spatial position of these catalytic residues determine that whether the hydrolysis will occur via overall

retention or via overall inversion of the anomeric configuration. There are exception in which the acetamido group at C-2 of the substrate acts as catalytic nucleophile not the amino acid residue of the enzyme (Terwisscha van Scheltinga et al. 1995). Hydrolysis of polysaccharides by PLs is completely different from GHs. Polysaccharide lyases act on anionic polysaccharides by a β elimination mechanism. The β elimination mechanism is accomplished in a three-stage reaction. In first stage, the carboxyl group of the substrate form a salt bridge with a positively charged amino acyl side chain in the active site of the enzyme which leads to the neutralization of substrate carboxyl group. In second stage, formation of a resonance-stabilized enolate intermediate occurs by a base-catalyzed abstraction of the proton at C-5 of the uronic acid. Finally, formation of a double bond between C-4 and C-5, which is possible after transfer of electrons from the carboxyl group, leads to the elimination of the 4-O-glycosidic bond and the formation of 4-deoxy-L-erythro-hex-4- enopyranosyluronate. This reaction generates a new non-reducing end containing an unsaturated uronate (Michaud et al. 2003). PLs and GHs receive considerable increase in their degradative potential from the appended CBM and the role of CBMs in the degradation of polysaccharides by GHs or by PLs cannot be ignored. CBMs plays three general roles with respect to the function of their associated catalytic modules i.e. a proximity effect, a targeting function and, a disruptive function (Boraston et al. 2004). To maintain the proximity of enzymes with their substrate polysaccharides, CBMs concentrate enzymes on the surface of the substrate facilitating rapid degradation of the polysaccharides by their cognate catalytic modules (Bolam et al. 1998). This is supported by the fact that proteolytic excision or genetic truncation of CBMs from the catalytic modules results in significant decreases in the potential of enzymes for insoluble polysaccharides degradation (Tomme et al. 1988; Bolam et al. 1998; Hall et al. 1995). Furthermore, CBMs classified in different groups based on their binding pattern with the

substrate. CBMs belonging to Type A modules bind to the surfaces of crystalline polysaccharides and can be appended to a variety of glycoside hydrolases. In contrary, Type B CBMs interact with single polysaccharide chains and are appended to only specific catalytic modules (Boraston et al. 2004). In addition to proximity and targeting functions, few reports suggests the disruptive functions by CBMs. This was first time observed in the CBM of Cellulase (Cel6A) from *Cellulomonas fimi* which mediate non-catalytic disruption of the crystalline structure of cellulose and enhanced the degradative capacity of the catalytic module (Din et al. 1994). Similar effect have only been observed for other cellulose-binding CBM (Gao et al. 2001).

2.10 Significance of the present study

As polysaccharide-degrading enzymes and oligosaccharide produced by them have wide application in various industry, biotechnology and medicine. Considerable focus has been put by the research groups from different countries over the decades to study the various parameters related to production of polysaccharide-degrading enzymes from microbes. To fulfil the industrial demand, Polysaccharide-degrading enzymes have been purified from various marine bacteria, however, the yield of the purified polysaccharide-degrading enzymes is very low. For example the yield of purified β agarase from *Microbulbifer* strain CMC-5, *Alteromonas* sp. C-1, and *Pseudomonas atlantica* were 0.2, 4.31, 0.22 mg/l respectively (Jonnadula and Ghadi, 2010, Leon et al., 1992, Morrice et al., 1983). In another report, 0.32 mg/l of α-agarase was purified from *Alteromonas agarolyticus* strain GJlB (Potin et al., 1993). Similarly the yield of *κ*-carrageenase from *Pseudoalteromonas* sp AJ5-13 is quite low i.e. 0.21 mg/l (Ma et al. 2010). Additionally, only 2.1 mg/l alginate lyase was purified from the culture free supernatant of *Vibrio* sp. SY08 (Li et al. 2016). Since polysaccharide-degrading enzymes can be exploited in production of oligosaccharides that demonstrate various therapeutic

property, it would be ideal to obtain these enzymes in higher yield with their proper native conformation. Cloning of polysaccharide degrading genes and their over expression in appropriate bacterial host system would be ideal to increase the yield of enzyme. Higher yield of enzymes have been obtained in many reported literature by cloning and expression of respective genes. For example, the yield of recombinant agarases from *Microbulbifer*-like JAMB A94 (Ohta et al., 2004), *Agarivorans* sp. JAMB-A11 (Ohta et al., 2005), *Microbulbifer thermotolerans* JAMB-A94 (Ohta et al., 2004), and *Microbulbifer* sp JAMB-A7 (Ohta et al., 2004) were 80, 51, 87, and 65 mg/l, respectively. *Microbulbifer* sp. have been reported to produce several types of polysaccharide-degrading enzymes (Gonzalez et al., 1997; Yoon et al., 2003; Tanaka et al., 2003). However only few of these genes have been cloned and expressed in *E.coli* host system (Swift et al. 2014; Ohta et al., 2004; Ohta et al., 2005). *Microbulbifer* strain CMC-5 and DD-13^T have been reported to produce several types of polysaccharide-degrading enzymes (Jonnadula et al., 2009; Vashist et al., 2013). Therefore, the objective of the present study was to clone and express the polysaccharide-degrading gene from selected the *Microbulbifer* sp.

Genomic library of an organism is the potent source for screening and obtaining recombinant clones with desired gene that can be used for expression studies. In order to obtain the clone of polysaccharide-degrading genes of selected *Microbulbifer* sp., preparation of genomic library was selected as one of the objective in the present study. Furthermore, cloning of the selected gene in expression vector was undertaken with a purpose to obtain polysaccharide degrading enzyme at higher yield that can be subsequently evaluated for application studies in the field of medical and industrial biotechnology. Cloning and expression of the gene along with the suitable affinity tags would also help in enzyme purification. Therefore, purification of one of the

polysaccharide-degrading enzyme and characterization of their biochemical property was selected as the second objective. Additionally, the cloned gene would be re-sequence and characterized using bioinformatics tools. Thus, the third objective of the present study was bioinformatics analysis of the cloned polysaccharide-degrading gene.

Analysis of Shotgun Genomic Library/Next Generation Sequencing Library of *Microbulbifer* sp. and Identification of Polysaccharide-Degrading Genes

This chapter describe the strategy and methodology followed for the preparation of shotgun genomic library of *Microbulbifer mangrovi* DD-13^{T} and *Microbulbifer* strain CMC-5. The clones from the shotgun genomic library from these *Microbulbifer* sp. were screened for polysaccharide-degrading activities. Additionally, the rationale, methodology and results obtained for the next generation sequencing (NGS) library of *Microbulbifer mangrovi* DD-13^{T} is also discussed. The genome sequence of DD-13^{T} is annotated with the special emphasis on the identification of polysaccharide-degrading genes. The results of genome annotation and polysaccharide-degrading genes identified from *Microbulbifer mangrovi* DD-13^{T} is presented in this chapter. A brief discussion at the end of the chapter is compares the results obtained during the present study with other reported relevant literatures to highlight the novelty of the present study.

3.1 Materials

Restriction enzymes, Calf Intestinal Alkaline Phosphatase (CIAP), T4 DNA ligase and DNA molecular weight markers were obtained from GeNei, Bangalore, India. Agarose, LB broth, LB agar, Alginate (sodium salt, polyguluronic and polymannuronic acid mixture), agar (purified, bacteriological), carrageenan (mixture from Irish moss), chitin (from crab shells), phenol red, congo red and Zobell Marine broth (ZMB) were purchased from HiMedia, Mumbai, India. All other reagents used were of AR grade.

3.2 Bacterial cultures

3.2.1 *Microbulbifer mangrovi* DD13^{T} (KCTC 23483)

The *Microbulbifer mangrovi* DD13^{T} used in present study was obtained from the departmental culture collection. It was isolated previously from mangroves of Goa, India (Vashist et al., 2013). The strain DD13^{T} degrades eleven different polysaccharides namely agar, alginate, chitin, carboxymethyl cellulose (CMC), laminarin, pectin,

pullulan, starch, carrageenan, β-glucan and xylan. Bacterial strain DD-13^T was routinely grown and maintained on Zobell Marine Agar (ZMA) plate.

3.2.2 *Microbulbifer* strain CMC-5 (MTCC 9889)

It is a multiple polysaccharide-degrading bacteria isolated from decomposing seaweeds of Anjuna coast, Goa (Jonnadula et al., 2009). The strain CMC-5 degrades six different polysaccharides *viz.* agar, alginate, carrageenan, CMC, xylan, and chitin. The strain CMC-5 was routinely grown and maintained on artificial sea water (ASW) agar plate or ASW broth.

3.2.3 Bacterial host used for DNA transformation

E.coli DH5α was used as host for transforming shotgun genomic library. The bacterial strain DH5α was maintained on LB agar plate. Furthermore, for expression of polysaccharide-degrading genes, *E.coli* BL-21(DE3) and *E.coli* BL-21(DE3)-Codon plus cells were used. These *E.coli* strains were maintained on LB agar plate containing tetracycline (15 µg/ml) and chloramphenicol (20 µg/ml) respectively.

3.3 Methodology

3.3.1 Isolation of genomic DNA from *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5.

Genomic DNA from bacterial strain DD-13^T and strain CMC-5 was isolated as mentioned elsewhere (Maloy, 1990). Briefly, the bacterial strains were grown in ZMB for 24 h at 30℃. After 24 h of incubation, the cells were recovered by centrifugation at 10,000 rpm and 4 ºC for 10 minutes. The bacterial cell pellet was resuspended in 1.5 ml of TE buffer. 10% SDS and proteinase K was added and the tube was incubated at 37 ºC for 1 h. After 1 h, mixture was extracted with equal volume of 1:1 phenol/chloroform.

Upper aqueous layer was taken after centrifugation. Nucleic acid was precipitated using sodium acetatae and ethanol. Pellet was washed with 70% ethanol and dissolved in 10 mM Tris-Cl (pH 8.0). RNAase treatment was done and the mixture was again extracted with phenol/chloroform. DNA was precipitated and washed with 70% ethanol, air dried and dissolved in 10 mM Tris-Cl (pH 8.0).

3.3.2 Restriction digestion of genomic DNA of *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5

Genomic DNA (1.0 µg) of *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5 were digested with *Sau*3AI and *Eco*RI respectively. The partial digestion reaction of genomic DNA of strain DD-13^T was optimised to obtain the genomic DNA fragment of ≤ 5 Kb by using the different concentration of *Sau*3AI (0.120U – 0.166U) and keeping the remaining component and conditions of digestion reaction constant. Similarly, the genomic DNA of *Microbulbifer* strain CMC-5 was partially digested using *Eco*RI. After completion of digestion, *Sau*3A1 and *Eco*RI were inactivated by heating at 65℃ for 10 minutes. The size of the digested genomic DNA fragment was analysed on 0.8 % agarose gel. The appropriately digested genomic DNA mixture was purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Germany) and used as insert for ligation reaction. Concentration of insert was determined using Qubit 2.0 flourometer (Invitrogen, USA).

3.3.3 Dephosphorylation of linearized pUC-18

The pUC-18 was linearized by the *Bam*HI and *Eco*RI separately. After completion of digestion reaction, *Bam*HI and *Eco*RI was inactivated by heating at 65℃ for 10 minutes. Linear pUC-18 was purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Germany). The linear purified

pUC-18 was treated with calf intestinal alkaline phosphatase (CIAP) to dephosphorylate the pUC-18. The linear and dephosphorylated pUC-18 plasmid was further purified using QIAquick gel extraction kit and used as cloning vector for ligation reaction. The concentration of vector was determined using Qubit 2.0 flourometer (Invitrogen, USA).

3.3.4 Ligation and transformation

The vector and insert were into ratio of 1:1, 1:6 and 6:1 for ligation. The reaction mixture was incubated for 3 h at 25 ºC followed by overnight incubation at 16 ºC. The ligase was inactivated by incubating the ligation reaction at 65℃ for 10 minutes and then stored at 4℃ in refrigerator until future use. The $CaCl_2$ induced competent *E.coli* DH5α cells were transformed with ligation mixture (50 ng of DNA) as per the standard protocols (Sambrook and Russel, 2001). The transformants were plated on LB agar plate containing 100 μg/ml of ampicillin and X-gal/IPTG. The recombinants were screened by blue/white screening assay.

3.3.5 Screening of recombinant clones for polysaccharide-degrading activity

Using sterile toothpicks, recombinant clones were patched on LB agar plate containing 0.5 % of individual polysaccharides *viz.* alginate, carrageenan and chitin. The bacterial clones were allowed to grow for 48 h at 37 ˚C. Polysaccharide-degrading activity related to agar, alginate, carrageenan and chitin would be evident from the formation of craters or zone of clearance around the bacterial colony. Furthermore, to visualize the zone of clearance around the bacterial colonies, the plates were flooded by various dyes/precipitating agents and incubated for 20 min. Lugol's iodine, cetylpyridinium chloride phenol red and congo red were used for the detection of agar, alginate and carrageenan and chitin degradation respectively (Hodgson and Chater, 1981; Gacesa and Wusteman, 1990; Ruijssenaar and Hartsman, 2001).

3.3.6 NGS library preparation and whole genome sequencing of *Microbulbifer mangrovi* DD-13^T

A single colony of *Microbulbifer mangrovi* DD-13^T was inoculated in ZMB (HiMedia, Mumbai, India) and grown at 30 °C for 24 h on an orbital shaker. The genomic DNA was isolated using GeNeiPure bacterial DNA isolation kit (Bangalore Genei, India) as per the manufacturer's instruction. The quantity and quality of the DNA were checked by Qubit 2.0 Fluorimeter (Invitrogen, USA) and NanoDrop (Eppendorff, USA). For next generation sequencing, a genomic library was prepared from strain DD-13^T. The Agilent SureSelect adapter was added to the DNA fragments, and the sequence was read on an Illumina NextSeq 500 platform.

3.3.7 Bioinformatics analysis of NGS sequence

The obtained raw reads were quality filtered using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with stringent filtering criteria. Low-quality reads with ambiguous sequences BN^ were removed. Finally, the reads with Q <30 bases were removed. The quality-filtered reads were assembled into contigs using SOAPdenovo v 1.05 (http://soap.genomics.org.cn/soapdenovo.html) with the default settings except K-mer values. After calculating different K-mer sizes, 31-mer yielded the best assembly. Gene prediction was performed with Glimmer v. 3.02 and Prodigal v. 2.5 (Delcher et al. 1999; Hyatt et al. 2010). The functional annotation of the gene products was achieved by BLAST analysis using the NCBI-nr protein database. The ribosomal RNA (rRNA) and transfer RNA (tRNA) genes were identified using RNAmmer 1.2 (Lagesen et al. 2007) and tRNAscan-SE 1.21 (Lowe and Eddy 1997), respectively. The cluster of orthologous groups was assigned to the predicted gene by analyzing the amino

acid sequence in COG database (http://www.ncbi.nlm.nih.gov/COG) using a WebMGA server that utilizes rps-BLAST 2.2.15 (Altschul et al. 1990).

3.3.8 Identification of polysaccharide-degrading Genes

The result of the BLAST search of all predicted genes was imported in an Excel sheet, and the polysaccharide-degrading genes were manually selected. Gene coordinates obtained by Glimmer v3.02 for the polysaccharide-degrading genes were fetched from the genome of strain DD-13^T and individually analysed by blastx and blastp against NCBI protein database (nr).

3.4 Results

3.4.1 Screening of genomic library of *Microbulbifer* sp. for polysaccharide-degrading activity

As observed from Fig 3.1a the partially digested genomic DNA of strain DD-13^T treated with 0.166 U of *Sau*3AI was observed to be < 3 Kb (Lane 2). Further, restriction digestion with 0.332 U of *Sau*3AI yielded fragments < 2 Kb (Fig 3.1a, lane 3). Thus with increasing enzyme concentration, the size of digested fragments decreased (lane 4, 5 & 6 of Fig 3.1a). Therefore, in order to obtain the DNA fragments of ~5 kb, the concentration of *Sau*3AI was further decreased. Optimal digestion of genomic DNA of strain DD-13^T was observed with 0.08 U of *Sau*3AI generating fragment sizes around 5 kb (Fig 3.1b, lane 3). Using the above optimised concentration of *Sau*3AI, DD-13^T genomic DNA was digested and subsequently purified using QIAquick Gel Extraction kit (Qiagen). The DNA fragments were used as insert for the ligation reaction. Similarly, the genomic DNA of strain CMC-5 was digested by *Eco*RI. Fig. 3.2 depicts the profile of digested genomic DNA of strain CMC-5. The digested CMC-5 genomic DNA was purified using QIAquick Gel Extraction kit (Qiagen) and was used as insert for ligation reaction.

Fig 3.3 depicts the profile of linear pUC-18 in comparison to undigested pUC-18. Furthermore, to minimize the self-ligation of linearized pUC-18 during ligation reaction, the linear pUC-18 was treated with Calf intestinal alkaline phosphatase. The efficiency of dephosphorylation was checked by ligating the dephosphorylated linear pUC-18. The dephosphorylated linearized pUC-18 comigrated with untreated linearized pUC-18 even after ligase treatment indicating the efficiency of dephosphorylation step (Fig 3.4). The linear dephosphorylated pUC-18 was further purified using QIAquick Gel Extraction kit (Qiagen) and used as vector for ligation reaction. The plasmid pUC-18 was linearized by *Bam*HI and used as vector for ligating *Sau*3AI digested fragments of DD-13^T genomic DNA whereas linearized plasmid pUC-18 with *Eco*RI end was used as vector for ligating *Eco*RI digested fragments from CMC-5 genomic DNA.

The ligation mixture was used to transform *E.coli* DH5α. The highest transformation efficiency was obtained when vector and insert were ligated in ratio of 6:1(Table 3.1). More than 60,000 clones were obtained during the library preparation. Fig 3.5a and Fig 3.6b shows a representative plates of recombinant clones obtained from the genomic library of strain DD-13^T and strain CMC-5 respectively using blue white screening assay.

Approximately 60,000 clones were screened for the degradation of agar, alginate, carrageenan and chitin using various dyes and precipitants. Fig 3.6(a-d) shows the representation of how clones were screened for the degradation capability of tested polysaccharides. Initially clones no. 109, 904, 933 and 937 from the genomic library of strain CMC-5 were selected for degradation of agar on the basis of clearance zone observed after spreading Lugol’s idodine (Fig 3.7). However, during subsequent subculturing of clone no. 109, 904, 933 and 937 did not demonstrate clearance zone after spreading Lugol’s iodine. This possibly might be due to instability of clones resulting in

deletion of fragment from pUC-18 (Godiska et al., 2010. Leach and Lindsey, 1986; Malagaon and Aguilera, 1998). No clones were obtained that degraded alginate, carrageenan and chitin from the genomic library of both strain DD-13^{T} and CMC-5.

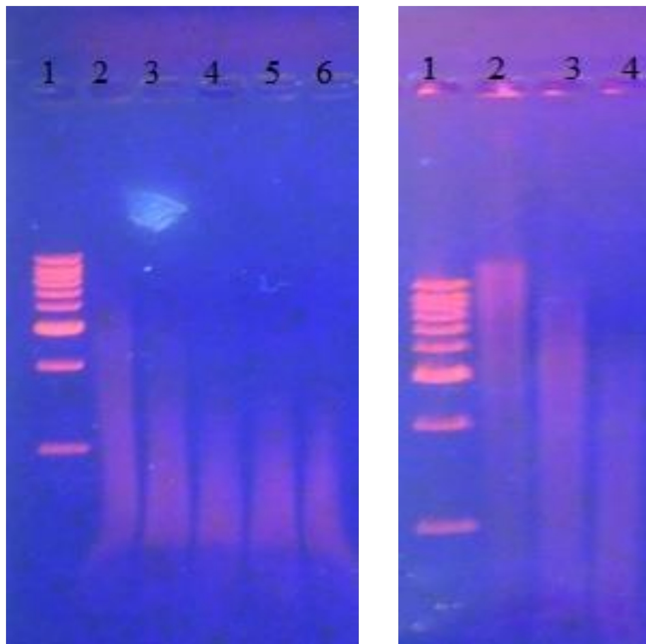


Fig 3.1a   Fig 3.1b

Fig 3.1 a & b: Genomic DNA of *Microbulbifer mangrovi* DD-13^{T} partially digested with *Sau*3AI

Fig 3.1a
Lane 1: 1 Kb ladder
Lane 2: Treatment with 0.166 U
Lane 3: Treatment with 0.332 U
Lane 4: Treatment with 0.498 U
Lane 5: Treatment with 0.664 U
Lane 6: Treatment with 0.830 U

Fig 3.1b
Lane 1: 1 Kb ladder
Lane 2: Treatment with 0.04 U
Lane 3: Treatment with 0.08 U
Lane 4: Treatment with 0.12 U

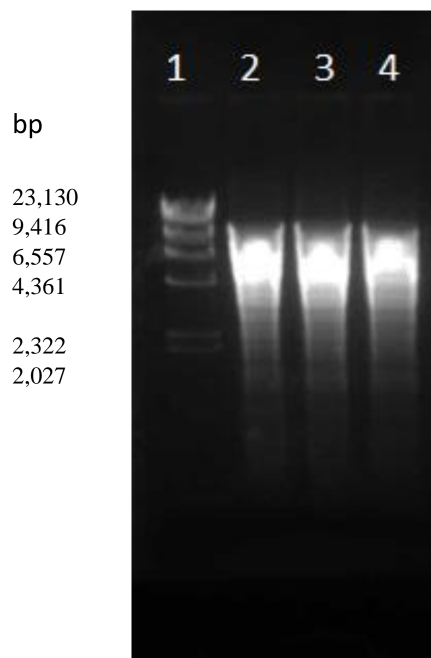


Fig 3.2

Fig 3.2: Genomic DNA of *Microbulbifer* strain CMC-5 digested with *Eco*RI

Lane 1: λ DNA/*Hind*III ladder
Lane 2, 3 & 4: Genomic DNA/*Eco*RI (20 U) digest

Fig 3.3: Restriction digestion of plasmid pUC-18 by *Eco*RI

Lane 1: Undigested pUC -18
Lane 2: pUC-18/*Eco*RI

Fig 3.4: Restriction digestion of plasmid pUC-18 by *Bam*HI

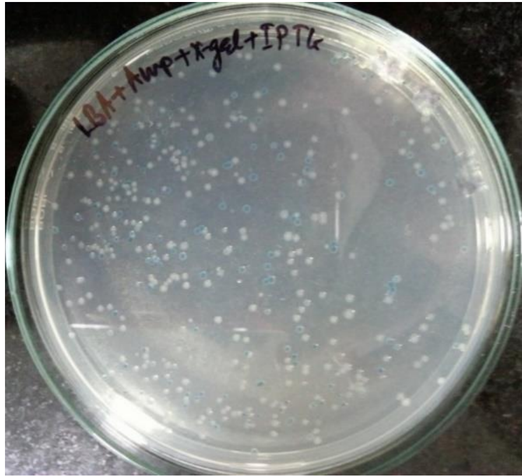
Lane 1: pUC-18 undigested
Lane 2: pUC-18 digested by *Bam*HI
Lane 3: linear pUC-18/Ligase
Lane 4: linear, dephosphorylated pUC-18/ ligase

Fig 3.3

Fig 3.4

Table 3.1: Details of various components used in ligation mixture and respective transformation efficiency

Components	Volume (µl)	Concentration	pmoles of DNA	Transformation efficiency
		A. Vector: Insert= 1:1		
Vector	1.3	45.5	0.025 pmoles	0.8×10^5 Transformants/µg of DNA
Insert	1.7	85.5	0.026 pmoles	
10X Ligase buffer	1.0	1X	-	
T4 DNA Ligase	0.5	1 U	-	
Sterile Milli Q	5.5	-	-	
Total volume	10.0	-	-	
		B. Vector: Insert= 1:5		
Vector	0.6	14.0	0.008 pmoles	0.2×10^5 Transformants/µg of DNA
Insert	2.6	130.0	0.039 pmoles	
10X Ligase buffer	1.0	1X	-	
T4 DNA Ligase	0.5	1 U	-	
Sterile Milli Q	5.3	-	-	
Total volume	10.0	-	-	
		C. Vector: Insert= 6:1		
Vector	2.5	87.5	0.048 pmoles	0.3×10^6 Transformants/µg of DNA
Insert	0.5	25.5	0.008 pmoles	
10X Ligase buffer	1.0	1X	-	
T4 DNA Ligase	0.5	1 U	-	
Sterile Milli Q	5.5	-	-	
Total volume	10.0	-	-	



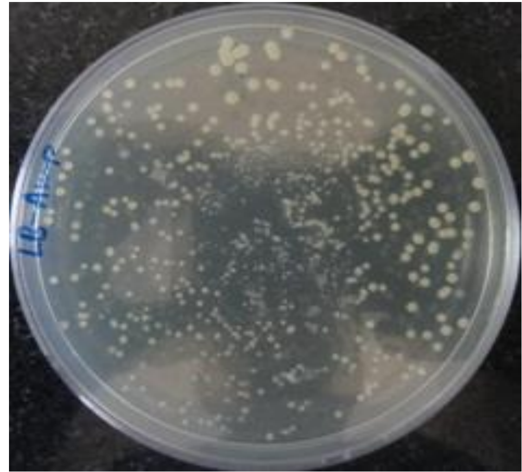
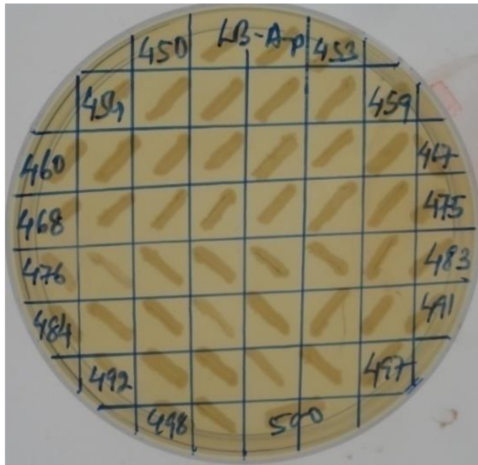


Fig 3.5 (a) **Fig 3.5 (b)**

Fig 3.5: Representative plate depicting the transformants obtained during preparation of genomic library from (a) *Microbulbifer mangrovi* DD-13^{T} and (b) *Microbulbifer* strain CMC-5



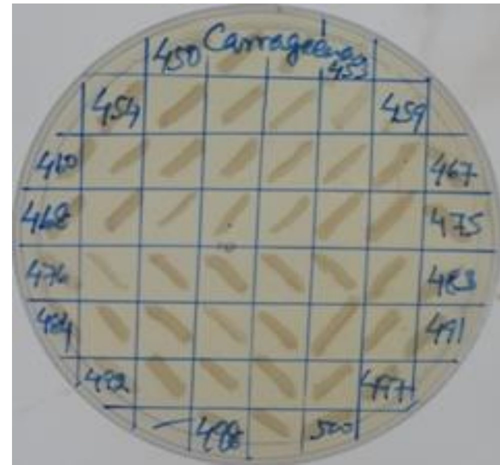
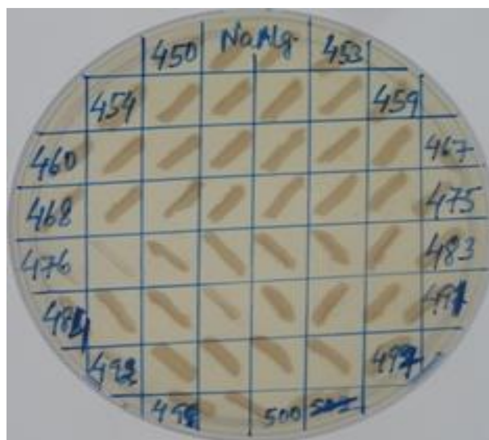


Fig 3.6 (a) **Fig 3.6 (b)**



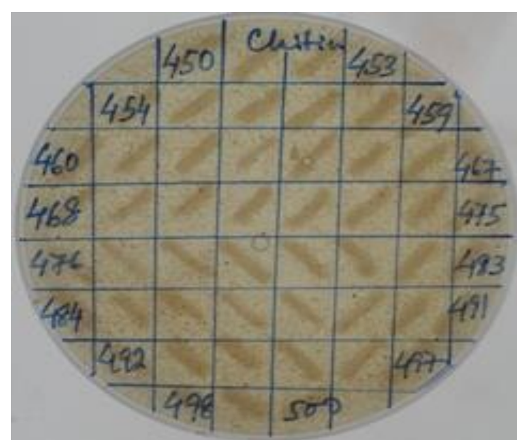


Fig 3.6 (c) **Fig 3.6 (d)**

Fig 3.6: Screening of clones from genomic library for polysaccharide-degradation on medium containing (a) agar (b) carrageenan (c) alginate, and (d) chitin

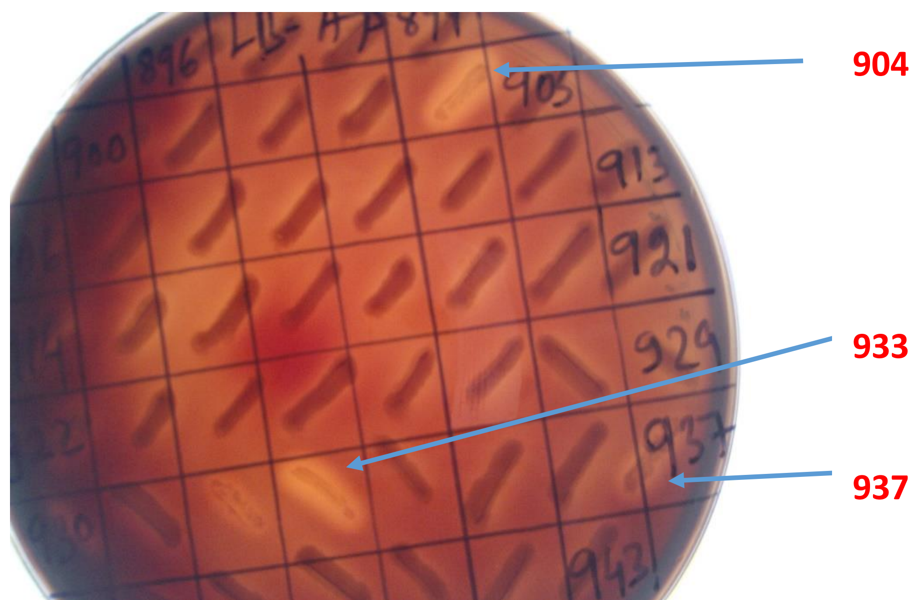


Fig 3.7: Detection of agarolytic activity in clones 904, 933 and 937 spreading Lugol's Iodine

3.4.2 Assembly of raw NGS library and quality analysis

A library of DNA fragments from the genome of DD-13^{T} was prepared by shotgun strategy and sequenced using the Illumina NextSeq 500 platform. A total of 6862280 reads with an average length of 151 nucleotides were obtained (Table 3.2). The total obtained reads were quality filtered with stringent filtering criteria. Quality-filtered paired-end reads were assembled using the SOAPdenovo alignment tool (http://soap.genomics.org.cn/soapdenovo.html). The assembly resulted into a total of 370 longer contigs with an average contig length of 7075 bp (Table 3.3). These 370 contigs together represents the draft genome of strain DD-13^{T}. The largest contig length was 153,024 bp. This was the best result obtained with a K-mer size of 31. The draft genome of DD-13^{T} is of 4,528,106 bp with a GC content of 57.15% (Table 3.3)

Table 3.2: Quality control data of genome sequencing of *Microbulbifer mangrovi* DD-13^T

Sr. No		Forward strand (R1)	Reverse strand (R2)
1	Total number of bases	1036204280	1036204280
2	Total number of reads	6862280	6862280
3	% bases >= Q 20	94.72 %	85.30 %
4	% bases >= Q 30	92.05 %	79.93 %
5	Average read length	151	151
6	Maximum read length	151	151
7	Minimum read length	151	151
8	Number of bases A	207737144	196903564
9	Number of bases T	181620625	163883609
10	Number of bases G	403328414	472173130
11	Number of bases C	243515834	202945939
12	Number of bases N	2263	298038
13	GC Content %	62.42	65.15

Table 3.3: Fundamental features of *Microbulbifer mangrovi* DD-13^T genome

	Category	Number	% of total
1	Total number of contigs	370	--
2	Total number of bases (Assembly size)	4528106	100
3	DNA coding number of bases (bp)	4457629	98.44
4	DNA G+C number of bases	2587717	57.15
5	DNA scaffolds	37	--
6	Total number of genes predicted	3749	100
7	Protein coding sequences (including hypothetical proteins)	3488	93.03
8	Genes in COGs	3348	89.3
9	Total number of RNA genes	37	0.99
10	rRNA genes		
11	5S rRNA	1	0.03
12	16S rRNA	1	0.03
13	23S rRNA	1	0.03
14	tRNA genes	34	0.91

3.4.3 Annotation of *Microbulbifer mangrovi* DD-13^T genome

A total of 3749 ORFs were predicted in the genome of strain DD-13^T. Out of 3749 ORFs, 3488 (93.0%) are protein-coding genes that demonstrated similarity with proteins in the NCBI nr-database with an e-value of <1e^{-5}. The analysis indicates that the 3488 candidate genes of strain DD-13^T have a predicted function and 922 (24.6%) were

identified to code for hypothetical proteins whereas 261 (6.96%) predicted proteins did not demonstrate any similarity with any other proteins from the NCBI nr-protein database. The Clusters of Orthologous Groups of proteins (COGs) database has been popular for functional annotation of proteins of newly sequenced genomes (Tatusov et al. 2001). Thus, based on similarity to COGs, 3348 genes (89.3%) of strain DD-13^T were functionally annotated (Table 3.4). In addition to protein-encoding genes, 34 tRNA and 3 rRNA genes were also predicted in strain DD-13 (Table 3.3)

Table 3.4: Functional classification of genes of *Microbulbifer mangrovi* DD-13 based on similarity with COGs

COG class	Total count (Percentage) *	Description
A	2 (0.06%)	RNA processing and modification
B	1 (0.03%)	Chromatin structure and dynamics
C	224 (6.69%)	Energy production and conversion
D	33 (0.99%)	Cell cycle control, cell division, chromosome partitioning
E	270 (8.06%)	Amino acid transport and metabolism
F	70 (2.09%)	Nucleotide transport and metabolism
G	212 (6.33%)	Carbohydrate transport and metabolism
H	157 (4.69%)	Coenzyme transport and metabolism
I	123 (3.67%)	Lipid transport and metabolism
J	182 (5.44%)	Translation, ribosomal structure and biogenesis
K	210 (6.27%)	Transcription
L	130 (3.88%)	Replication, recombination and repair
M	237 (7.08%)	Cell wall/membrane/envelope biogenesis
N	48 (1.43%)	Cell motility
O	154 (4.60%)	Posttranslational modification, protein turnover, chaperones
P	220 (6.57%)	Inorganic ion transport and metabolism
Q	65 (1.94%)	Secondary metabolites biosynthesis, transport and catabolism
R	403 (12.04%)	General function prediction only
S	295 (8.81%)	Function unknown
T	145 (4.33%)	Signal transduction mechanisms
U	107 (3.20%)	Intracellular trafficking, secretion, and vesicular transport
V	59 (1.76%)	Defense mechanisms
Z	1 (0.03%)	Cytoskeleton

*Percentage has been calculated based on total number of functionally assigned genes (3348)

3.4.4 Identification of polysaccharide-degrading genes from the assembled genome sequence of *Microbulbifer mangrovi* DD-13^T

The polysaccharide-degrading genes were identified based on the homology of DD-13^T genes with nr-protein database of NCBI using BLASTx with stringent criteria. Only those homology matches that had an e-value of $<10^{-5}$ and a query coverage of 99% was selected. Furthermore, the BLASTx result was confirmed/counter checked by analysing the amino acid sequence using the BLASTp. The presence of conserved domain related to the particular polysaccharide-degrading genes/enzymes were also taken into consideration while considering the DD-13^T nucleotide/amino acid sequence as a polysaccharide-degrading genes/enzyme. Our analysis reveals the presence of a wide range of polysaccharide-degrading genes in the genome of strain DD-13^T that degraded various polysaccharides of seaweeds and plants origin. In DD-13^T genome, five agarase, two alginate lyase and one carrageenase gene were detected. Additionally, four genes for chitinase, two xylanase, one pullulanase, five amylase, three pectate lyase, five arylsulfatase and three β-glucosidase genes were detected. Furthermore, the DD-13^T genome encodes singular gene for many other polysaccharide-degrading enzymes including α-glucuronidase, exo-poly-α-d-galacturonosidase, endo-1,4-d-glucanase, β-glucanase, 1,4-β-d-xylanxylohydrolase, glucan-1,4-α-glucosidase, β-mannosidase, α-l-fucosidase, cellobiohydrolase, putative glucoamylase I, xyloglucanase, β-glucosidase, α-glucosidase, β-glycosidase, arabinan endo-1,5-α-l-arabinosidase, and α-N-arabinofuranosidase. The contig wise position of the identified polysaccharide-degrading genes of strain DD-13^T is shown in the table 3.5.

Table 3.5: Polysaccharide-degrading genes identified in the *Microbulbifer mangrovi* DD-13^T genome and position of the genes in the DD-13^T genome.

Sr. No.	Contig no. and position of gene	Annotation of Polysaccharide degrading genes	Total number of genes detected
1	contig1094:14877-18161 contig1270:133540-135879 contig1270:148758-151022 contig908:82-1140 contig908:1201-3651	Agarase	5
2	contig1246:14949-17225 contig1246:17247-19490	Alginate lyase	2
3	contig1270:97064-101125	Carrageenase	1
4	contig1176:1453-4569 contig1212:35745-36755 contig1270:61090-62316 contig1270:62760-65579	Chitinase	4
5	contig884:105-2843 contig1090:9803-11326	Xylanase	2
6	contig1082:12386-14932	Pullulanase	1
7	contig1204:24298-26052 contig1230:35822-37561 contig1230:14935-16878 contig1230:7994-10270 contig1090:9803-11326	Amylase	5
8	contig1240:21784-23556 contig1240:27163-31128 contig1240:14289-15569	Pectate lyase	3
9	contig1024:3158-4504 contig1116:17660-19456 contig1260:36351-37820	Glycoside hydrolase	3
10	contig1058:6291-8525	Glycoside hydrolase family 16 domain-containing protein (lichenase, agarase, kappa-carrageenase, endo-β-1,3-glucanase, endo-β-1,3-1,4-glucanase, endo-β-galactosidase	1
11	contig1186:19531-20925	Glycoside hydrolase family 5 (endoglucanase, endomannanase, exoglucanases, exomannanases, β-glucosidase and β-mannosidase)	1
12	contig1104:10922-12475	Glycoside hydrolase family 43 (α-L-arabinofuranosidases, endo-α-L-arabinanases, β-D-	1

		xylosidases and exo α-1,3-galactanase)	
13	contig1270:76325-77794	Glycoside hydrolase family protein	1
14	contig1150:13661-15196 contig1150:15278-17521 contig1150:19821-21356 contig1270:73306-74748 contig1270:117398-118831	Arylsulfatase	5
15	contig1066:5042-7222	α-glucuronidase	1
16	contig1240:47262-48638	Exo-poly-α-D-galacturonosidase	1
17	contig1218:31916-32779	Endo-1,4-D-glucanase	1
18	contig1266:72943-74391	β - glucanase	1
19	contig1236:44604-46211	1,4 β-D-xylanxylohydrolase	1
20	contig1104:2594-19458	Glucan 1,4-α-glucosidase	1
21	contig1036:3363-4805	β-mannosidase	1
22	contig1158:23994-26477	α - L fucosidase	1
23	contig1186:21070-22869	Cellobiohydrolase	1
24	contig1034:8599-9165	Putative glucoamylase I	1
25	contig1036:105-3311	Xyloglucanase	1
26	contig1058:15-1493 contig1102:14996-16333 contig1266:72943-74391	β -glucosidase	3
27	contig1158:12819-14477	α-glucosidase	1
28	contig990:4017-5705	β-glycosidase	1
29	contig1010:7339-8685	Arabinan endo-1,5-α-L-arabinosidase	1
30	contig1110:9285-10262	α-N-arabinofuranosidase	1

3. 5 Discussion

Genomic library of an organism is the potential source of obtaining a desired clone of that organism. In present study, genomic library of the two multiple polysaccharide-degrading bacteria namely *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5 were prepared with an objective to obtain the clone with polysaccharide-degrading gene. In previous reports, researchers have used the genomic library of polysaccharide-degrading bacteria made by similar strategy like present study as a source of obtaining the clones of polysaccharide-degrading genes. The clones of β-agarase gene have been obtained from the genomic libraries of *Vibrio* sp. strain V134, *Pseudoalteromonas* sp. CY24, *Microbulbifer*-like isolate and *Pseudomonas* sp. SK38 (Zhang & Sun, 2007; Ma et al., 2007; Ohta et al., 2004; Kang et al., 2003). In another report, clone of α-agarase gene has been screened from the genomic library of *Alteromonas agarilytica* (Flament et al., 2007). Similarly alginate lyase gene have been isolated from the genomic library of *Sphingomonas* sp. MJ-3 (Park et al., 2012). Furthermore the κ-carrageenase and λ-carrageenase genes were obtained from the genomic library of *Cytophaga drobachiensis* and *Pseudomonas carrageenovora* respectively (Barbeyron et al., 1998; Guibet et al., 2007). Similarly, chitinase genes have been also cloned using genomic library strategy. The clones of chitinase genes of *Alteromonas* sp. strain O-7 and *Vibrio harveyi* have been obtained from their genomic libraries respectively (Tujibo et al., 1993; Svitil et al., 1997).

Various dyes/ precipitants has been widely used for the screening and detection of polysaccharide-degradation capability of microorganisms (Hodgson and Chater, 1981; Gacesa, 1992; Ruijssenaar and Hartsman, 2001). In present study, functional screening of genomic library of *Microbulbifer* sp. was done to detect the polysaccharide-degrading clones using dyes/precipitants based plate assay. However, we fail to detect the any

polysaccharide-degrading clones from the genomic library of *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5. This may be because of the following limitations of the dye/precipitants based screening system used for the detection of polysaccharide-degradation capability. Dye based screening assay could only detect the extracellularly produced enzymes activity however the foreign gene product in *E.coli* host may or may not secretes extracellularly. Secondly, as the genomic library was made in plasmid vector, the expression of the cloned gene may be very low which may not be detected/visualized by the dye based plate assay. Additionally, cloning of the DNA fragments into high copy number plasmid vector like pUC-18 is associated with the many difficulties that consequently prevent in-vitro expression and functional analysis of the cloned gene. Supercoiling of the plasmid pUC-18 can induce cruciform and other secondary structures favouring deletion or rearrangements (Godiska et al., 2010. Leach and Lindsey, 1986; Malagaon and Aguilera, 1998). Deletion/rearrangements of the cloned fragment might be the possible region of loss of agarolytic properties of the clone no. 109, 904, 933 and 937 from the genomic library of strain CMC-5. These clones had depicted agar degradation as evident from the observation of clearance zone around these clones on spreading Lugol's iodine. Later, in the experiments conducted to confirm/reproduce the agarolytic nature, these clones fail to produce the clearance zone. Moreover, the agar degrading clones form the craters or halos on LB agar plates that was further confirmed by the formation of halos of by lugol's iodine staining (Zhang & Sun, 2007; Kang et al. 2003). In present study none of the clones depicted craters or halos while the screening of clones from the genomic library of *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5. Alternatively, screening of the clones using advanced techniques like colony PCR or radio labelled probes or enzyme conjugate antibodies would have resulted in the detection of polysaccharide-degrading clones from

the genomic libraries of *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5. The alginate lyase gene was detected from the genomic library of *Sphingomonas* sp. MJ-3 using PCR probe based screening (Park et al., 2011). Similarly, the chitinase gene was screened from the genomic library of *Vibrio harveyi* using anti chitinase antibody (Svitil et al., 1997).

Therefore, with the objective of detecting the polysaccharide-degrading genes, whole genome of *Microbulbifer mangrovi* DD-13^T was sequenced. For whole genome sequencing, Next generation sequencing (NGS) library was made and the sequence were read on Illumina NextSeq 500 platform. The assembly of sequencing reads yielded the draft genome size of *Microbulbifer mangrovi* DD-13^T as 4,528,106 bp that is proximate to the reported draft genome size (4,223,108 bp) of *Microbulbifer elongatus* HZ11 (Sun et al. 2014). However the genome size of completely sequenced *Microbulbifer thermotolerans* DAU221 (3,938,396 bp) is comparatively less than DD-13^T genome size (Lee and Choi, 2016). The genome size of strain DD-13^T is also comparatively closer to the completely sequenced genome size of another multiple polysaccharide-degrading bacterium *Saccharophagus degradans* 2-40 that was earlier classified as *Microbulbifer degradans* (Weiner et al. 2008). The similar draft genome size of strain DD-13^T indicates that the genome sequence represents the almost full genome of strain DD-13^T. The GC content of strain DD-13^T is 57.15 % which is similar to *Microbulbifer* sp. namely *Microbulbifer elongatus* HZ11 (56.70 %), *Microbulbifer thermotolerans* DAU221 (56.57 %) (Sun et al., 2014; Lee and Choi, 2016).

Further genome annotation of *Microbulbifer mangrovi* DD-13^T reveals that, out of 3749 predicted ORFs, 3488 (93.0 %) are protein coding genes that is slightly higher than the number of genes reported from *Microbulbifer elongatus* HZ11 (3293) and *Microbulbifer thermotolerans* DAU221 (3301) indicating the functional diversity of

Microbulbifer mangrovi DD-13^T (Sun et al., 2014; Lee and Choi, 2016). Interestingly, our analysis further reveals that the 922 (24.6 %) genes of strain DD-13^T code for hypothetical proteins and 261 (6.96 %) predicted proteins did not demonstrate any similarity with any other proteins from the NCBI nr-protein database. Therefore, a total of approximately 30 % of predicted genes of strain DD-13^T are probable novel genes. This is supported by the facts that hypothetical proteins are known as novel prospective targets for future experimental studies (Galperin and Koonin 2004). This makes the strain DD-13^T, a very unique, potential bacterial strain for further studies and finding novel proteins of biotechnological and industrial applications.

As the main objective of the genome sequencing was to detect the polysaccharide-degrading genes, *Microbulbifer mangrovi* DD-13^T has the diverse range of polysaccharide-degrading genes. Out of 3488 protein coding genes, 55 (1.58 %) genes are devoted for the polysaccharide-degrading enzymes that makes the strain DD-13^T a potential strain for the degradation of seaweed and plant origin polysaccharides. The strain DD-13^T genome encodes five agarases that could possibly constitute the complete pathways for agar metabolism. The genus *Microbulbifer* is relatively younger and to the best of our knowledge besides the genome of *Microbulbifer mangrovi* DD-13^T few more *Microbulbifer* genomes are sequenced that includes *Microbulbifer elongatus* HZ11 (Sun et al., 2014), *Microbulbifer thermotolerans* DAU221 (Lee et al. 2016), *Microbulbifer agarilyticus* GP101 (Accession no. NZ_CP019650.1) and *Microbulbifer* sp. CCB-MM1 (Accession no. PRJNA305828). However, only *Microbulbifer elongatus* HZ11 genome has been annotated with the emphasis on the identification of polysaccharide-degrading genes. The *Microbulbifer elongatus* HZ11 has five agarases, nine alginate lyases, one cellulase and two amylase encoding genes (Sun et al., 2014). The present study indicates that the *Microbulbifer mangrovi* DD-13^T is the most diverse *Microbulbifer* sp. in terms

of polysaccharide degradation potential as it has many additional polysaccharide-degrading genes than *Microbulbifer elongatus* HZ11 including four genes for chitinase, two xylanase, one pullulanase, three pectate lyase and five arylsulfatase. Further, the versatility of DD-13^T for polysaccharide-degradation potential is attributed to the presence of many singular gene in the genome *viz.* α-glucuronidase, exo-poly-α-d-galacturonosidase, endo-1,4-d-glucanase, β-glucanase, 1,4-β-d-xylanxylohydrolase, glucan-1,4-α-glucosidase, β-mannosidase, α-l-fucosidase, cellobiohydrolase, putative glucoamylase I, xyloglucanase, β-glucosidase, α-glucosidase, β-glycosidase, arabinan endo-1,5-α-l-arabinosidase, and α-N-arabinofuranosidase. This genomic potential of strain DD-13^T to degrade complex polysaccharide enables the survival of the strain in mangrove ecosystem.

CHAPTER 4

Cloning and Expression of Pullulanase Gene of *Microbulbifer mangrovi* DD-13^{T} and Biochemical Characterization of Purified Pullulanase

The present chapter describe the cloning of the pullulanase gene of *Microbulbifer mangrovi* DD-13^T in pET-22b and their expression in *E.coli* Bl-21 (DE3). Further, the chapter focuses on the purification of recombinant pullulanase from *E.coli* Bl-21 (DE3) harbouring the pET-22b containing pullulanase gene from Microbulbifer mangrovi DD-13T. The chapter also elaborates the biochemical properties of the purified pullulanase. The final section of the chapter provides details on comparison of biochemical properties of purified recombinant pullulanase with pullulanase reported from other bacteria highlighting the novel properties of pullulanase of *Microbulbifer mangrovi* DD-13^T.

4.1 Materials

4.1.1 Enzymes and reagents

The restriction enzymes and T4 DNA ligase were obtained from Promega, USA. PR polymerase and DNA molecular weight ladder were obtained from GeNei, Bangalore, India. Bacterial genomic DNA isolation kit and plasmid purification kit were obtained from GeNei, Bangalore, India. His-tagged protein purification kit and pre-stained protein molecular weight marker were obtained from HiMedia, Mumbai, India. Pullulan (extrapure) was obtained from Sisco Research laboratories Pvt. Ltd (Mumbai, India). Acrylamide and Bis-acrylamide used were of molecular biology grade. The other chemical used in present study was of analytical grade (AR grade).

4.1.2 Plasmids and transformation host

The pET-22b was used as expression vector. *E.coli* DH5α was used as host for the propagation of pET-22b. The *E.coli* DH5α harbouring the pET-22b was maintained on LB-agar plate containing 100 µg/ml of ampicillin. The *E.coli* BL-21 (DE3) was used as transformation and expression host system and was maintained on LB agar plate containing 30 µg/ml of tetracycline.

4.2 Methodology

4.2.1 Analysis of pullulanase encoding ORF

As described in previous chapter, *Microbulbifer mangrovi* DD-13^T genome was sequenced and several polysaccharide-degrading genes was identified and included the pullulanase gene (contig1082:12386-14932). The nucleotide sequence of region contig1082:12386-14932 was screened for the presence of possible potential ORF using NCBI's ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The nucleotide sequence of the identified ORFs was translated into amino acid sequence using ExPasy translate tool (http://web.expasy.org/translate/). The amino acid sequence corresponding to the identified ORF was searched against NCBI's nr-protein database using BLASTp. The ORF depicted identity to the pullulanase from NCBI' nr-protein database and was further searched for the presence of conserved domain specific for the pullulanase using NCBI's conserved domain search server (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Finally the potential putative ORFs of pullulanase gene was selected for cloning and expression.

4.2.2 PCR primer designing

To clone the full ORF of pullulanase gene of *Microbulbifer mangrovi* DD-13^T in pET-22b, the PCR primer was designed using Custom primers-OligoPerfect™ Designer, Invitrogen (https://tools.thermofisher.com/content.cfm?pageid=9716) and the features of designed PCR primers was analysed using OligoAnalyser v3.1 (https://eu.idtdna.com/calc/analyzer). To the PCR primers, restriction site of *Hind*III and *Xho*I were introduced (Table 4.1). For the extended translation of cloned DNA fragment with his-tagged region of pET-22b, the stop codon of the pullulanase gene was not considered during PCR primer designing. The designed PCR primer was validated for

inframe cloning using Snapgene software (http://www.snapgene.com/). For this study, trail version of Snapgene was downloaded. In the snapgene software, nucleotide sequence of pullulanase gene from *Microbulbifer mangrovi* DD-13^T was imported and using the above designed PCR primers, *in-silico* PCR was performed. The PCR product was digested by *Hind*III and *Xho*I using the restriction digestion option in the software. Similarly, pET-22b was digested with *Hind*III and *Xho*I. Further, using the restriction cloning option of the snapgene software, *Hind*III and *Xho*I digested PCR product was cloned into the pET-22b pre-cut with the *Hind*III and *Xho*I. The inframe cloning was confirmed by analysing the cloned gene product using the ORF option in the software. After successful simulation, the PCR primer was synthesized at Shrimpex Pvt Ltd (Chennai, India). For the amplification of pullulanase gene, PAGE purified PCR primers were used.

Table 4.1: PCR primers designed for the amplification of pullulanase gene from *Microbulbifer mangrovi* DD-13^T

Position of gene	**PCR Primers sequence**	**length**	**Restriction site**
contig1082:12386-14932	F: 5'CGC**AAGCTT**ATGACCCAGGTTAGCCAG3' R: 5' ACT**CTCGAG**ATGGCGGTAAAGCACG 3'	27 25	*Hind*III *Xho*I

4.2.3 Amplification and cloning of pullulanase gene of *Microbulbifer mangrovi* DD-13^T

The genomic DNA of *Microbulbifer mangrovi* DD-13^T was isolated using GeNeiPure Bacterial DNA purification kit (GeNei, Bangalore, India). The quantity and quality of the genomic DNA was checked using Qubit 2.0 Flurimeter (Invitrogen, USA) and NanoDrop (Eppendorf, Germany) respectively. The genomic DNA of strain DD-13^T was used as template in PCR for the amplification of pullulanase gene. The pullulanase gene was amplified using the PR polymerase (GeNei, Bangalore, India). The various

components of the PCR reaction shown in table 4.2. In order to generate the restriction site of *Hind*III and *Xho*I in PCR product, two-stage cycling parameters were set during PCR. Initial 5 cycles were set with the annealing temperature 51℃ whereas the remaining 35 cycles were set with annealing temperature 59℃ (Table 4.3). The size of the amplified product was analysed on 1% agarose gel and compared with 1 Kb molecular weight marker. The PCR product having predicted amplicon size was purified using QIAqucik gel extraction kit (Qiagen, Germany).

Table 4.2: Various components used for PCR for the amplification of pullulanase gene of *Microbulbifer mangrovi* DD-13^T

Sr. No.	Components of PCR	Volume (µl)	Quantity
1	Sterile Milli Q water	13.0	-
2	10X PR Polymerase assay buffer	2.0	1X
3	10 mM dNTPs mix	2.0	1mM each
4	Genomic DNA of strain DD-13^T	0.5	50 ng
5	Forward primer	1.0	0.25 µM
6	Reverse primer	1.0	0.25 µM
7	PR Polymerase	0.5	1.5 U
8	Total volume	20.0	-

Table 4.3: Cycling parameters for the PCR amplification of pullulanase gene from *Microbulbifer mangrovi* DD-13^T

Sr. No.	Reaction	Temperature (℃)	Time	Number of cycles
1	Initial denaturation	94	4:00 min	1
2	Denaturation	94	30 sec	
3	Annealing	51	30 sec	5
4	Extension	72	2 min 30 sec	
5	Denaturation	94	30 sec	
6	Annealing	59	30 sec	35
7	Extension	72	2 min 30 sec	
8	Final extension	72	10 min	1

4.2.4: Restriction digestion, ligation and transformation

The purified PCR product was double digested with *Hind*III and *Xho*I. Restriction digestion was carried out in 20 µl reaction volume containing 0.5 µl (5 U) of *Hind*III/*Xho*I for ~ 1 µg of the purified amplified product. The reaction mix tube was incubated at 37℃ for 1 h. *Hind*III and *Xho*I was inactivated at 65℃ for 10 min. The digested mixture was purified using QIAqucik gel extraction kit (Qiagen, Germany). A fraction of purified digested PCR product was analysed on 0.8 % agarose gel. The concentration and purity of the digested purified PCR product was determined by Qubit 2.0 flurimeter and NanoDrop respectively. The digested PCR product was ligated to pET-22b pre-cut with the *Hind*III and *Xho*I. The ligation reaction was set in 1:3 and 1:5 vector insert ratio (Table 4.4). Using the ligation mixture, the $CaCl_2$ induced competent cells of *E.coli* Bl-21 (DE3) were transformed as per the standard protocol (Sambrook & Russell, 1989). Each transformants were manually screened for the recombinant plasmid by isolating and performing double digestion of the plasmid using *Hind*III and *Xho*I. As a result of double digestion with *Hind*III and *Xho*I, a plasmid gave approx. 2.5 Kb band (cloned insert) as well as a correspondent band for vector pET-22b from one of the transformants. The insert of one purified clone was re-sequenced using T7 sequencing primers. After confirmation by sequencing, true recombinant clone was designated as BL21-pET22b-Pull and chosen for the expression studies of pullulanase.

Table 4.4: Various components used during ligation reaction and the respective transformation efficiency

Sr. No.	Vector: Insert ratio	1:3		1:5	
	Reaction components	**Volume (µl)**	**Concentration**	**Volume (µl)**	**Concentration**
1	Nuclease free water	6.70	-	7.30	-
2	Vector (pET-22b)	1.14	50ng (0.014 pmols)	0.57	25.00 ng (0.007 pmol)
3	Insert (Pullulanase amplicon)	0.76	68.26 ng (0.043 pmols)	0.63	56.89 ng (0.036 pmol)
4	10X Ligase buffer	1.00	1 X	1.00	1 X
5	T4 DNA ligase	0.50	1.5 U	0.50	1.5 U
6	Total volume	10.00	-	10.00	-
	Transformation efficiency	~ $0.4X10^4$ Transformants/µg DNA		~ $0.7X10^4$ Transformants/µg DNA	

4.2.5 Induction of expression of pullulanase gene in *E.coli* Bl-21 (DE3)

BL21-pET22b-Pull was grown in LB broth containing 100 µg/ml of ampicillin at 37 ˚C on orbital shaker at 120 rpm. The *E.coli* Bl-21 (DE3) having pET-22b (BL21-pET22b) was also inoculated in LB broth and grown at 37 ˚C and was used as control. 500 µl of overnight grown cultures was further inoculated in 50 ml of LB broth containing 100 µg/ml of ampicillin and incubated at 37 ˚C on orbital shaker at 120 rpm. When the OD reached 0.5, a final concentration of 1 mM IPTG was added and the cells were further allowed to grow for 24 h at 18˚C on orbital shaker set at 120 rpm. The cells were harvested by centrifugation at 11000 rpm, 4˚C for 10 min. The cell pellet was re-suspended in ice-cold 5 ml of 20 mM Tris-Cl (pH 7.0) and sonicated (alternate cycle with 10 sec pulse/without pulse of 40 secs for six cycles) using Vibra Cell (Sonics & Materials INC. USA). The cell-debris free sonicated extract was collected by centrifugation at 11000 rpm, 4˚C for 10 min and stored at -20˚C. 20 µl of the sonicated extract was analysed on 12 % SDS PAGE.

4.2.6 Purification of recombinant pullulanase

The recombinant pullulanase was purified from BL21-pET22b-Pull using his-tagged protein purification kit as per the manufacturer's instructions (HiMedia, Mumbai, India). Briefly, the Ni-NTA agarose column was equilibrated with 10 mM imidazole. To the equilibrated column, 400 µl of cell-debris free sonicated extract was added and mixed. The column was stored at 4℃ for 30 minutes. After incubation, column was centrifuged at 600xg for 1 minute. The eluate was discarded. The spin column was washed thrice with 75 mM imidazole. The trapped his-tagged protein was eluted using 250 mM imidazole. A fraction of purified pullulanase was analysed on 12 % SDS PAGE.

4.2.7 Pullulanase assay

The pullulanase activity was determined by measuring the release of reducing sugar from the pullulan substrate by 3, 5-dinitrosalicylic acid assay (DNSA) method (Miller, 1959). Briefly, 50 µl of purified enzyme was added to the 950 µl of 0.1 % pullulan dissolved in 20 mM of Tris-Cl (pH 7.0) in a test tubes. The reaction tubes was incubated at 37℃ for 30 min. The reaction was stopped by adding 1 ml of DNSA reagent to the reaction tubes. The tubes were kept in boiling water bath for 10 minutes followed by cooling under tap water. The absorbance was measured at 540 nm using spectrophotometer (Shimadzu, Kyoto, Japan). All the reactions were set in triplicates and appropriate substrate and enzymes blanks were included in the assay. Glucose was used as reference sugar. One unit of enzyme was defined as release of 1µmole of glucose per minute from pullulan in specified assay condition.

4.2.8 Biochemical characterization of the purified recombinant pullulanase

4.2.8.1 Determination of optimum concentration for pullulanase assay

0.1% of pullulan dissolved in 50 mM Tris Cl (pH 7.0) was incubated with different concentration of purified recombinant pullulanase (0, 6.25, 12.5, 25.0 and 50µg) for 30 min at 37°C. Total reaction volume was made to 1.0 ml. After 30 min of incubation, the amount of reducing sugar released was quantified by DNSA method as described above. The reducing sugars released were estimated against glucose standard.

4.2.8.2 Optimum incubation period for pullulanase assay

A fixed concentration of purified recombinant pullulanase was added to 0.1% pullulan dissolved in 50 mM Tris-Cl (pH 7.0) at 37°C in 5 individual test tubes and incubated at different time intervals (10, 20, 30, 40 and 50 min). Total reaction volume was made to 1.0 ml with 50 mM Tris-Cl (pH 7.0) buffer. Reducing sugars released were determined by DNSA method described above.

4.2.8.3 Optimum pH for pullulanase activity

The activity of purified recombinant pullulanase was measured at different pH using various buffers. 50 mM citrate buffer (pH 4.0, 5.0 and 6.0), 50 mM Tris-Cl (pH 7.0 and 8.0) buffers were used for pullulanase assay. Pullulanase activity was measured in triplicates in a 1.0 ml of reaction volume containing purified enzyme and 0.1% pullulan, prepared in 50 mM citrate buffer (pH 4.0, 5.0 and 6.0) and 50 mM Tris-Cl (pH 7.0 and 8.0). The reaction mixture was incubated for 30 min at 37°C. Reducing sugars released were measured by DNSA method as described above.

4.2.8.4 pH stability of pullulanase

The purified pullulanase was incubated for 60 min in various buffers (pH 4.0, 5.0, 6.0, 7.0 and 8.0) buffers in triplicates. The 50 mM citrate buffer (pH 4.0, 5.0 and 6.0) and 50 mM Tris-Cl (pH 7.0 and 8.0) buffer were used. After the incubation, 0.1% pullulan dissolved in 50 mM Tris-CI (pH 7.0) was added to the reaction tubes containing the purified pullulanase and the volume was made to 1.0 ml. The reaction mixture was incubated at 37°C for 30 min. The reducing sugars released were measured by DNSA method as mentioned above.

4.2.8.5 Optimum temperature for pullulanase activity

Pullulanase activity was estimated in triplicates by incubating the purified enzyme with 0.1% pullulan dissolved in 50 mM Tris Cl (pH 7.0). Total reaction volume was made to 1.0 ml with 50 mM Tris CI (pH 7.0) buffer. The reaction mixture was incubated for 30 min at various temperatures (25, 30, 35, 40, 45, 50 and 55°C). Reducing sugars released were measured by DNSA method.

4.2.8.6 Thermal stability of pullulanase

The purified recombinant pullulanase was incubated for 60 min at 25, 30, 35, 40, 45, 50 and 55°C in triplicates. After the incubation, reaction tubes containing the enzyme were kept in ice for 10 min. 0.1% pullulan dissolved in 50 mM Tris CI (pH 7.0) was added to above enzyme fractions and the volume made to 1.0 ml with the same buffer. The reaction mixture was incubated at 45°C for 30 min. The reducing sugars released were measured by DNSA method as mentioned earlier.

4.2.8.7 Effect of metal ion/reagents on pullulanase activity

The purified recombinant pullulanase was incubated with 10 mM EDTA at 4°C for 1 h. The enzyme was later dialyzed against 50 mM Tris C1 (pH 7.0) for 24 h at 4°C and was used in the present study to determine the recovery of activity in presence of different cations/ reagents. Pullulanase activity (EDTA treated and dialyzed) was measured with 0.1% pullulan dissolved in 50 mM Tris C1 (pH 7.0) containing 2 mM of following metal salts/ reagents: $CaC1_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CuC1_2$, NaCl and KCl. The 0.1% pullulan substrate containing different metal ions or chemical agents was incubated with purified pullulanase at 45°C for 30 min. The reducing sugars released were measured by DNSA method as described earlier. Pullulanase activity observed with non- EDTA treated purified pullulanase was considered as 100% and relative activity of EDTA treated pullulanase as well as EDTA treated pullulanase amended with metal/ reagents was determined.

4.3 Results

4.3.1 Analysis of ORF for pullulanase

As mentioned earlier, the gene co-ordinate contig1082:12386-14932 obtained through GLIMMER was annotated as pullulanase encoding gene in *Microbulbifer mangrovi* DD-13^T. Further, the nucleotide sequence of the contig1082:12386-14932 was analysed to identify potential ORF. NCBI ORF finder predicted a longest ORF of 2415 bp starting from ATG (start codon) and ending with TAA (stop codon). The translated amino acids of the predicted ORF demonstrated identity with pullulanase from other Type species belonging to genus *Microbulbifer, Photobacterium, Pseudoalteromonas* etc. (Described in detail in chapter 5). Additionally, many conserved domains detected in the putative pullulanase of *Microbulbifer mangrovi* DD-13^T included a specific hit for the AmyAc_Pullulanase_LD-like domain (Accession no. cd11341) that is an α-amylase catalytic domain found in pullulanase indicating the identity of the ORF as pullulanase encoding ORF. Additionally, the of amino acid residues identified for the active site (Tyr^{374}, Asp^{375}, His^{422}, Arg^{477}, Asp^{479}, Leu^{480}, Glu^{510}, Trp^{512}, Val^{543}, Arg^{546}, His^{614}, Asp^{615}, Asn^{616}, His6^{74} and Tyr^{676}) and catalytic site (Asp^{479}, Glu^{510}, and Asp^{615}) of putative pullulanase of *Microbulbifer mangrovi* DD-13^T was conserved and is similar to other well characterized Type I pullulanase reported earlier, thus, endorsing the annotation as pullulanase encoding ORF (Wei et al., 2015). Thus, the ORF of 2415 bp is considered as the potential ORF for pullulanase and was selected for cloning and expression studies in heterologous host.

Before performing the cloning experiments, in-frame cloning of pullulanase gene was virtually simulated using the snapgene software. The simulation studies indicates the successful in-frame cloning of pullulanase gene in pET-22b. Further, the simulation

studies indicates that the translated product is a full length protein of expected size. Fig 4.1 depicts steps involved in generation of pullulanase clone (pET-22b-Pull)

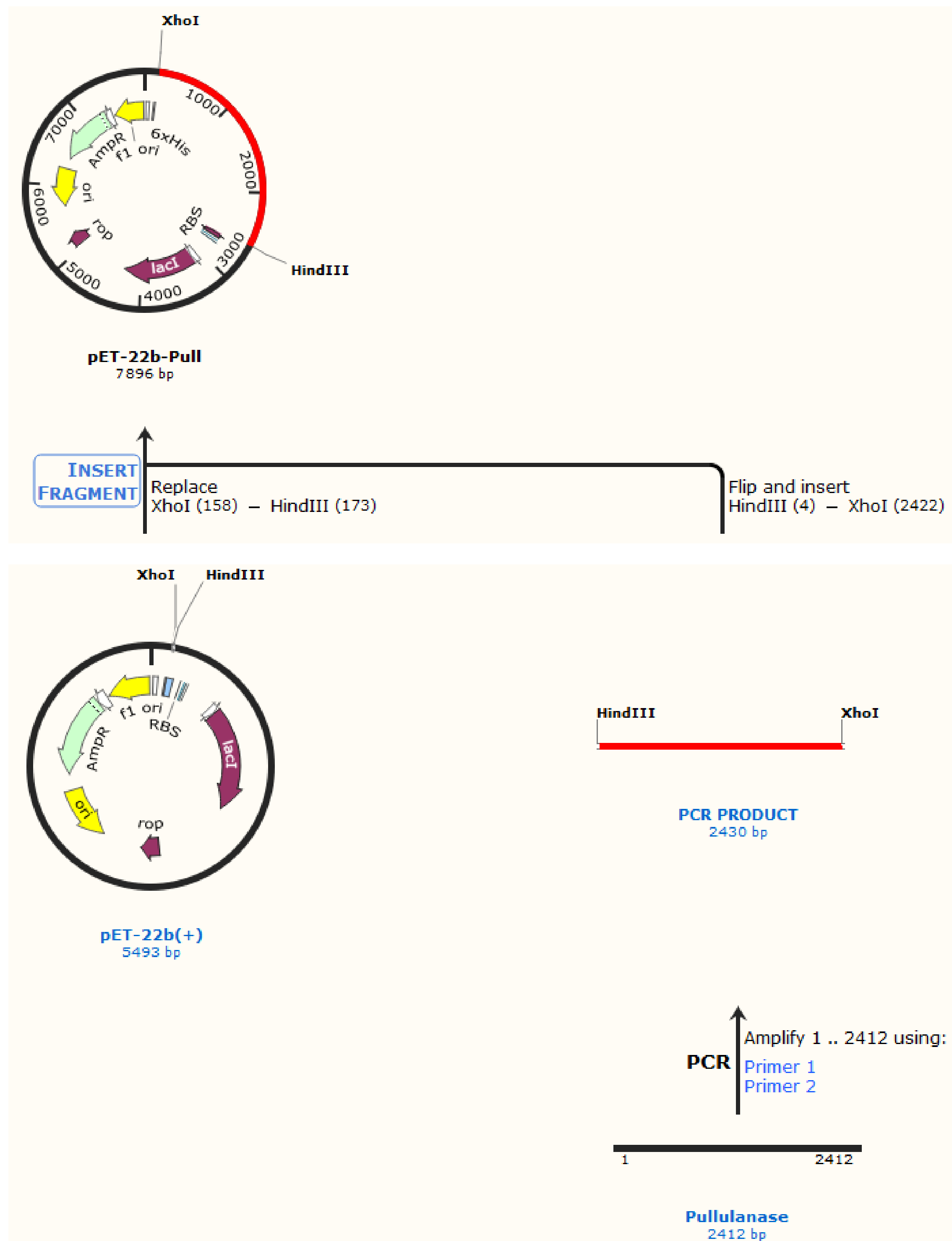


Fig 4.1: Depiction of cloning strategy of pullulanase gene from *Microbulbifer mangrovi* DD-13^{T} in to pET-22b. The figure has been generated using snapgene software (Trail version).

4.3.2 Cloning of pullulanase gene of *Microbulbifer mangrovi* DD-13^{T}

Using the high fidelity PR polymerase and genomic DNA of *Microbulbifer mangrovi* DD-13^{T} as a template, the pullulanase gene was amplified. The amplification of pullulanase gene was achieved by two step PCR. As the PCR primer designed for the amplification had two parts, the complimentary region (18 bp and 16 bp in forward and reverse primer respectively) and the 5' leader sequence containing the site for restriction enzymes and additional 3 nucleotides for facilitating the binding of restriction enzymes to the PCR product in further processing for the cloning. So, the annealing temperature for the initial five cycles was set at 51 ℃ allowing the annealing of only complementary region of the PCR primer to the template DNA resulting generation of new template that would have the additional nucleotide complementary to the 5' leader sequence of the PCR primers too. In next 35 cycles, the annealing temperature was set at 59℃ that would allow the binding of full length PCR primer (complementary region as well as 5' leader sequence of the PCR primer) to the template (generated in initial five cycles) and resulting in generation of restriction site for *Hind*III and *Xho*I in final PCR product. Fig 4.2 depicts the profile of amplified product of pullulanase gene. As evident from the agarose gel, the size of the amplified product of the pullulanase gene is approximately 2.5 kb (Fig 4.2) and is in agreement with the predicted size indicating the specific amplification. The amplified product was purified using QIAquick gel extraction kit (Qiagen, Germany). Further, the purified pullulanase amplicon was subjected to double digestion by *Hind*III and *Xho*I. Again the digested pullulanase amplicon was purified using QIAquick gel extraction kit (Qiagen, Germany) and the DNA concentration was determined. The concentration was observed to be 90.0 ng/µl.

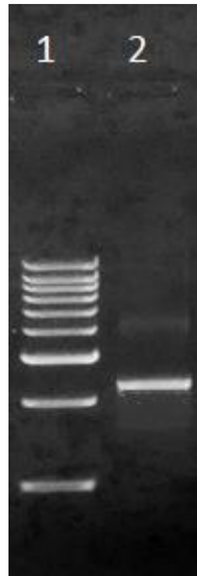


Fig 4.2: Profile of amplified product of pullulanase gene from *Microbulbifer mangrovi* DD-13^T

Lane 1: 1 Kb ladder
Lane 2: Amplified product

Fig 4.2

The pET-22b was digested with *Hind*III and *Xho*I. Fig 4.3 depicts the profile of pET-22b double digested with *Hind*III and *Xho*I. The size of pET-22b is 5449 bp which is also evident from the agarose gel profile of pET-22b digested with *Hind*III and *Xho*I (Fig 4.3). The *Hind*III and *Xho*I digested pET-22b was purified using QIAquick gel extraction kit (Qiagen, Germany). The concentration of linear pET-22b was found to be 44.0 ng/μl.

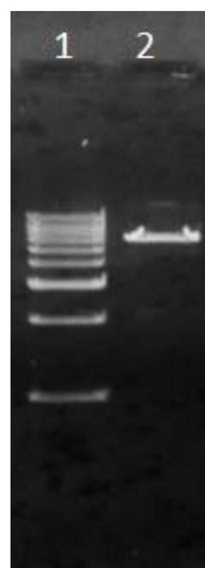


Fig 4.3: pET-22b digested by *Hind*III & *Xho*I

Lane 1: 1 Kb DNA ladder
Lane 2: Linear pET-22b

Fig 4.3

The *Hind*III and *Xho*I digested pullulanase gene amplicon was ligated with *Hind*III and *Xho*I linearized pET-22b at 1:3 and 1:5 ratio (Table 4.4). 5µl (~ 60 ng of total DNA for 1:3 and ~ 40 ng for 1:3 ligation reaction) of ligation mixture was used to transformed the $CaCl_2$ induced competent *E.coli* Bl-21 (DE3) cells. Transformation efficiencies of 10^4 transformants/µg of DNA was obtained. The Fig 4.4 shows the representative plate showing the colonies obtained for the transformation.

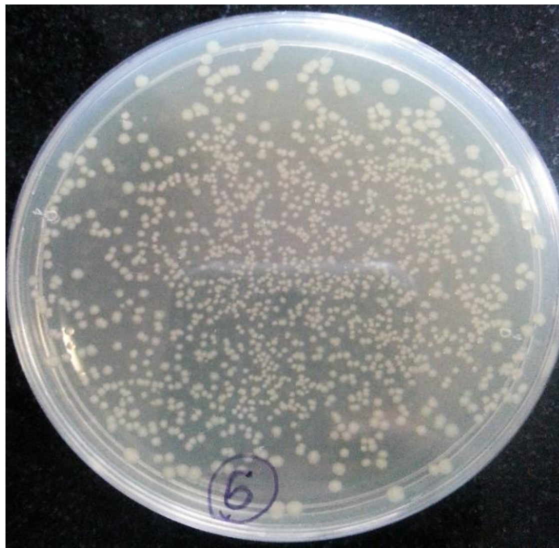
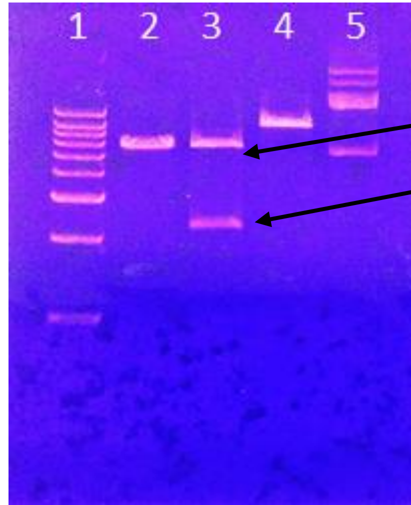


Fig 4.4: Representative plate showing transformants obtained for the cloning of pullulanase gene in pET-22b

Fig 4.4

The obtained transformants were screened for the recombinant plasmid by the double digestion of the plasmid isolated from the transformants. Fig 4.5 depicts the profile of double digested recombinant plasmid using *Hind*III and *Xho*I. The agarose gel profile depicts the presence of two DNA bands, one that co-migrated with linear pET-22b whereas the molecular weight of other DNA band is approximately 2.5 Kb. The former band is for pET vector used during the ligation reaction whereas the later DNA band is for pullulanase amplicon which clearly indicates that the pullulanase encoding DNA fragments has been successfully cloned in pET-22b. The same has been confirmed by the resequencing of the cloned DNA fragments using the T7 sequencing primers (Fig 4.6) (Sequencing data analysis will be discussed in chapter 5). The clone that gave 2.5

Kb band for insert confirmed by sequence analysis found prefect by sequence analysis was designated as pET-22b-Pull construct. The *E.coli* Bl-21 (DE3) harbouring the pET-22b-Pull construct was used for expression studies of pullulanase gene of *Microbulbifer mangrovi* DD-13^{T}.



Vector DNA (pET-22b)

Insert DNA band (Pullulanase amplicon)

Fig 4.5: Profile of double digested recombinant plasmid by *Hind*III and *Xho*I

Lane 1: 1 kb DNA Ladder
Lane 2: Linear pET-22b (Control)
Lane 3: Recombinant clone digested with *Hind*III & *Xho*I
Lane 4: Linear recombinant clone

Fig 4.5

>Pullulanase from *Microbulbifer mangrovi* DD-13^{T}
ATGACCCAGGTTAGCCAGAACGCATTTCACACCTGCCAGTCGTTCGCCAACGGCGATAGCAGCGGCGGCCCCCGCTT
CAAGGTGGATCGTCACGGCGACTGGAGCGAAAGCTATCCCACTGCAGATTACGCCGTCACCGGCAACCGCTCCTACG
AAATCACCTTTCAAAGCGACAGCCTCAACATCGCTGTCAATGAAGTGGCGAGCTGCGATGAAGGCAGCGTCCATGGC
GCCAATTTTTCCTCACTCTATTTTCGCGGAACACCCAACACATGGAACGCCTCTTCCATGAATCTGGTGGCCGACAAT
ACCTGGCAACTGCAGATCCACTTTGACGGCCAGGCAAACCAGCGGTTCAAGCTCGATGTGTTCGGTGACTGGAGCCA
GAATTACGGTGATAACAACAGCGACGGCATCCTGGATCAAACCGGTAGTGATATTTATACGGACGTGGTCGGCGATT
ACCTGCTAACGGTCAACGATGCGAACCTCAACTACACTCTGGAACCGGTTGAATGCACCGAAAACTGCAATCAAAGC
CTGGATACCCTGGGTGCAGTGTACTCCTCAACGGAAACCACTTTCTCCCTGTGGTCTCCAGATCACAACGACGTACAA
CTGGTTCTCGATGGCCAGAATTATCCGATGGAAAAGATTCCCGACGCTGCGCTTGCCGCACAGGGGATGACAGACAT
CTATTCGGTTACCGTAAGCGGCGACTGGAAACTGAAGCCCTATTATTTTGTCGTCAATGGCGTAGTGGTCCGCGATCC
TTACGGCAAGATGGTGGAACCCGATAGCGATAACAATATCGTCATGGATATGTCCGCTACAGAGTTACCCAACGGAT
GGTCCGCACGCCCTTTACTCAATGAGCGTGAGGACGCGGTAATCTACGAGGTGCACGTACGCGATTTCACTATTGCA
CCCGAGTCCGGTGTCAGCAGTGACAAGCGCGGAAAATTTCTCGGCATGGTCGAAAGCGGTAGCCGTTTCAATGGCGT
GGCGACGGGTATCGACCACCTGAAGGAACTCGGTGTGACGCATGTGCAATTGCTACCCGTGTATGACTTCAATGCCT
GCGCCGACCCTGCCGACACTGCCTGTTACAGCTGGGGTTACGACCCGCGCAATTTCAACGTACCGGAAGAACGCTAC
TCGCAGACACCCACGGACTACGAAAATCGCGTAAGCGAATTCAAGACCATGGTCGACGAGTTTCACAAAGCCGGTAT
TCGAGTCATTATGGATGTGGTCTACAACCACACTTATTCGAAATCTGTTTTCGAGCCAATATCCAGCAGCTACTACAC
CCCAACCGACCTGTCCGGCACTGGCAACAGTATTGATGCCAATGTCCCCATGGTCGGCCGCATGATCCGCGACTCCCT
AGAATATTGGGTAGATGAATACAATATTGACGGTTTTCGTTTTGACCTGATCGGTATTTTTGACTATGACGAAGTGGC
CGATTGGGCGGACAACCTGAATAGCAAATTTCCGGACCGCAACCTGTTGATCTACGGCGAACCCTGGAACGGCTTTG
CGGCAGACTCGCGCCAGCCGGAGCGCGTGCGCTTGGGCACCATCGGCCGTATACACGAGTCTCACGCCGGGGTGTTC
AACCCGAAATTTCGTGAAGCCATCAAAGGACAAAACGATAACGGCGGCTGCAACCCCGGCGACTGCTTCGCTCTCAA
TAACAATCCCGATACCTGGCGAATCGAAGTGGGCAGCCGCGGCGGTCTCCGCTACACAAAAGACAAGGATACGGCC
ATCGACACCTGGGACCCGATGTTTGCGATGGATCCGGAACAAAGCATCAACTACGTCTCGGCCCACGATAACCTGAC
CTTGCGGGACAAGATCCTGCAATGGGCAGACTTGAATGGCATCAGCAGGGATAGCGGCTACTTGCGAAGAATCCAG
ATGTTCGCCAACGGGATTGTTCTAACCAGCCAGGGTATTCCATTCCTGCACGGCGGTGTCGAACTCATGCGCGACAA
GCAGGAGGATCACAACAGCTACGATTCGCCGGACGCAATCAACCAGTATTACTGGCAGTGGAAAATCGACAATGCT
GATATCTACGCGTACTACAAAGATGTGATTGCACTACGCCGCGCGCACCCCGCTTTCCGGCTTACCAGCTGGGATGC
AATCGACCAGCACATGACCAGCAACCGGCCACGCTACGGTATTGTAGTCCATCACATTGATGGCGCTGCGGTAGGTG
ACAGCTGGAGTGAAGTTATTGTGATCTACAACAGTGCGGACAATTACACCTACAGCTTGCCCGCGGGCGAGTGGAAA
GTGGCACTGGAAAAATCAGACCCCGCTGCGGGTAACGGACGCAGCGTGAGTGGCTCCGTGGTGGCAGAGGGGACCG
CGGTTACCGTGCTTTACCGCCATTAA

Fig 4.6: Nucleotide sequence of pullulanase gene from *Microbulbifer mangrovi* DD-13^{T}

4.3.3 Expression of recombinant pullulanase gene

The induced expression of recombinant pullulanase gene was acheived by adding 1 mM IPTG to the early log growth of phase BL21-pET22b-Pull recombinant culture. BL21-pET22b-Pull was grown at three different temperature (37 ℃, 25 ℃ and 18 ℃) to determine the expression level of recombinant pullulanase gene. The expression of pullulanase gene was not observed at 25 ℃ and 37 ℃. Additionally, the sonicated extract of BL21-pET22b-Pull grown on 25 ℃ and 37 ℃ did not showed pullulanase activity. Although, pullulanase activity was not observed in the sonicated extract of BL21-pET22b-Pull grown at 18℃ for 3 h, the pullulanase activity was observed in the sonicated extract of BL21-pET22b-Pull grown at 18 ℃ for 24 h. The SDS PAGE profile of these fraction is shown in the Fig 4.7. Thus growth of early log phase BL21-pET22b-Pull at 18 ˚C for 24 h in the presence of 1 mM IPTG is ideal for expression of the pullulanase gene of *Microbulbifer mangrovi* DD-13^T in *E.coli* BL-21 (DE3).

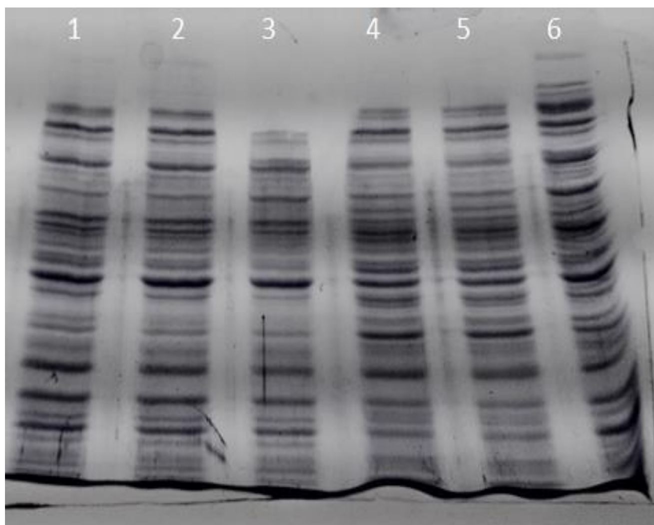


Fig 4.7: SDS PAGE profile of proteins from BL21-pET22b-Pull grown at 18 ℃ for different period
Lane 1: control *E.coli* BL-21 (DE3) harbouring pET-22b
Lane 2: BL21-pET22b-Pull without IPTG (3h)
Lane 3: BL21-pET22b-Pull with IPTG (3h)
Lane 4: control *E.coli* BL-21 (DE3) harbouring pET-22b
Lane 5: BL21-pET22b-Pull without IPTG (24 h)
Lane 6: BL21-pET22b-Pull with IPTG (24 h)

4.3.4 Purification of pullulanase from BL21-pET22b-Pull

The recombinant pullulanase from BL21-pET22b-Pull was purified to homogeneity using Ni-NTA agarose protein purification kit. About 2.4 mg recombinant pullulanase was purified up to 6.54 folds with a recovery of 62.84 % by Ni-NTA affinity chromatography (Table 4.5). The crude extract enzyme specific activity was 4.69 U/mg whereas enzyme purified using Ni-NTA agarose column has specific activity up to 30.7 U/mg. However no enzyme activity was observed in the fraction obtained from untransformed *E.coli*. The purified enzyme migrated on SDS-PAGE as a single band with an apparent molecular mass of about 85.0 kDa (Fig 4.8a) which is closer to the predicted molecular weight of the pullulanase i.e. 90 KDa. Additionally, purified pullulanase demonstrated a single band on native PAGE also (Fig 4.8b). The results indicated that Ni-NTA affinity chromatography was an appropriate method for the protein purification (Table 4.5). Further, to confirm the identity of purified pullulanase, the protein was recovered from native PAGE and the pullulanase activity was determined in the recovered fraction by DNSA method. Observation of pullulanase activity (1.18±0.02 U/ml) in the protein recovered from native PAGE confirming the identity of purified protein as pullulanase. The equal amount of native PAGE slice not having the protein was taken and subjected to protein recovery, consider as control. However no pullulanase activity was observed in the control.

Table 4.5: Purification of pullulanase from *E.coli* BL-21(DE3) carrying the pET-22b-Pull construct

Purification Step purification	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	% yield	Fold
Crude Extract	117.25	25	4.69	100	1.00
Ni-NTA	73.68	2.4	30.7	62.84	6.54

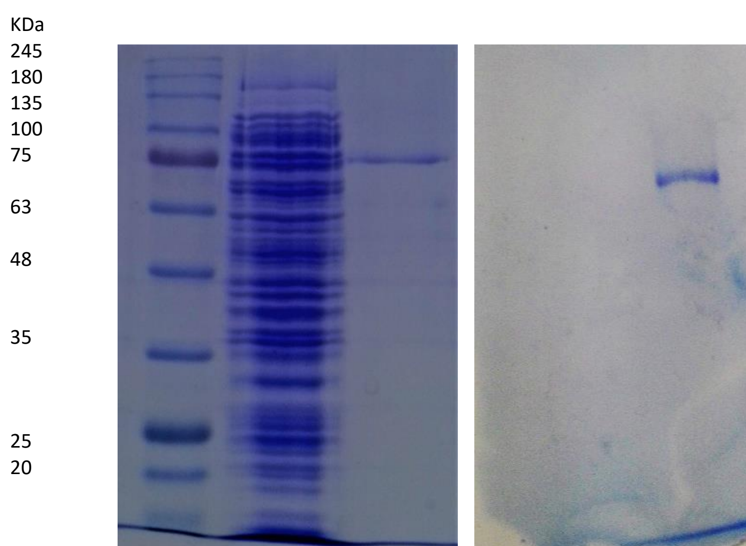


Fig 4.8a **Fig 4.8b**

Fig 4.8: SDS PAGE and native PAGE profile of purified pullulanase

Fig 4.8a

Lane 1: Protein molecular weight marker
Lane 2: Crude protein
Lane 3: Purified pullulanase

Fig 4.8b: Purified pullulanase on native PAGE

4.3.5 Biochemical characterization of pullulanase

The concentration of the purified pullulanase, incubation period for assay, optimum pH and stability, optimum temperature and thermal stability, effect of various agents after EDTA treatment and for the optimum pullulanase activity were determined by DNSA method as described in methodology section. One unit of the enzyme activity was defined as the amount which liberates 1 μmol of reducing sugar per minute in the assay condition.

4.3.5.1 Optimum concentration for pullulanase assay

In order to determine an optimal concentration of pullulanase for biochemical characterization various concentration (6.25, 12.5, 25 and 50 μg) of the purified pullulanase were evaluated for the release of reducing sugar. As depicted from Fig 4.9, the purified pullulanase activity profile showed linearity upto 12.5 μg of purified pullulanase protein concentration. At higher concentration of the protein, the pullulanase activity showed saturation. 12.5 μg of the purified recombinant pullulanase was hence further selected for carrying biochemical characterization of purified pullulanase.

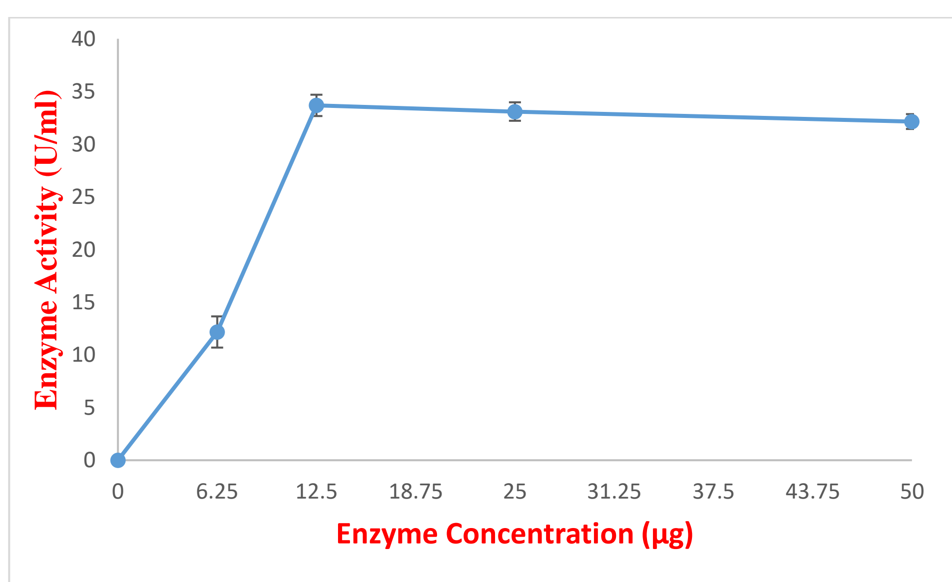


Fig 4.9: Determination of optimum concentration of pullulanase for the assay

4.3.5.2 Optimum incubation period for pullulanase assay

To determine the optimal incubation time to perform assay of pullulanase from *Microbulbifer mangrovi* strain DD-13^T, 12.5 µg of purified pullulanase was incubated with the substrate at different incubation time with pullulan and reducing sugar released were quantified. The pullulanase activity was observed to depict linear trend up to 30 min of incubation after which activity reached a plateau (Fig 4.10). As depicted from the Fig 4.7, 30 min was selected as an ideal incubation time for performing the pullulanase assay.

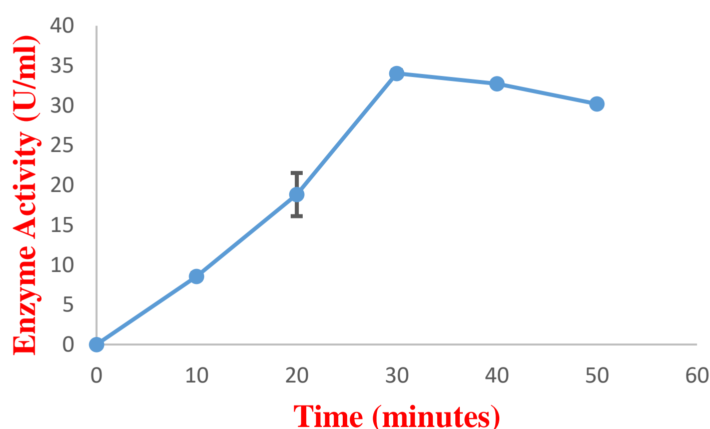


Fig 4.10: Determination of optimal incubation period for pullulanase assay

4.3.5.3 Optimum pH and pH stability for the pullulanase

Optimum pH for pullulanase activity was determined using different buffer systems. The pullulanase activity at different pH is shown in Fig 4.11. Pullulanase activity was observed between pH 5 to 7 and the enzyme showed low activity at pH 4 whereas a sharp decrease in pullulanase activity observed at pH 8. The optimum pullulanase activity was observed at pH 7.0. The pullulanase from *Microbulbifer mangrovi* strain DD-13^T was stable in the pH range of 5.0 to 7.0 for 1 h**,** however pullulanase retain approx. 50 % activity after 1h at pH 4 and pH 8 (Fig 4.11).

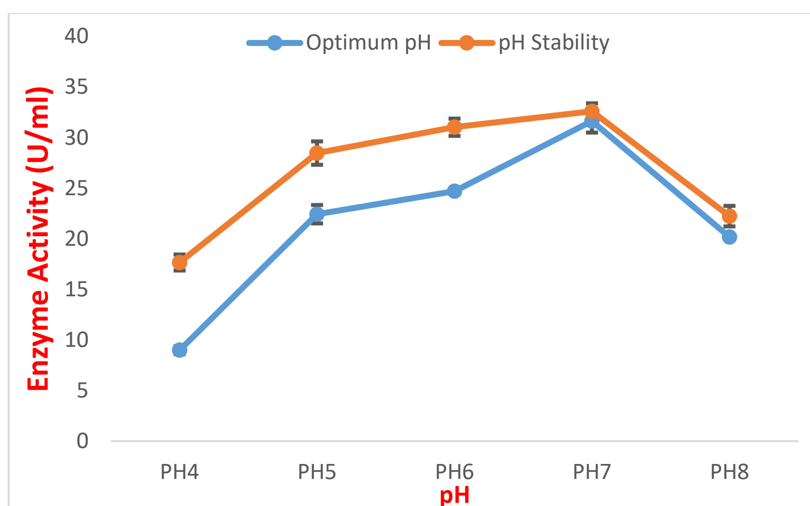


Fig 4.11: Optimum pH for pullulanase activity and pH stability

4.3.5.4 Optimum temperature and temperature stability

The profile of pullulanase activity at various temperatures is as shown in Fig 4.12. The optimal temperature for the pullulanase activity was observed to be 45°C. As observed from Fig 4.12, the enzyme was partially active in the temperature range of 30 to 50°C. The enzyme stability was measured at various temperatures for 60 min. The recombinant pullulanase from *Microbulbifer mangrovi* DD-13^{T} remains partially active at 25℃ and start losing activity gradually 25 - 45°C and became totally inactive at 50°C for 1 h.

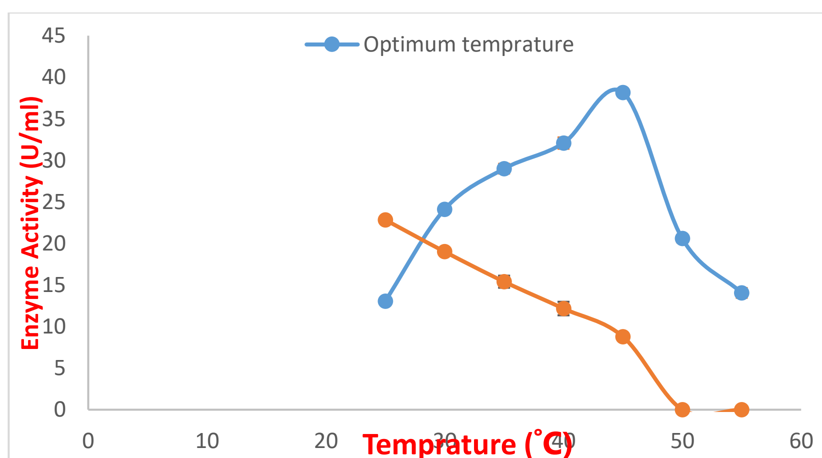


Fig 4.12: Optimal temperature and thermal stability

4.3.5.5 Effect of metal ions/chemical reagents

After 10 mM EDTA treatment, the purified recombinant pullulanase completely lost its activity in comparison to the control untreated pullulanase. Metal ions were tested to recover the pullulanase activity of EDTA treated pullulanase at a concentration of 2 mM. 42 % activity was recovered by Ca and Zn ions whereas Fe and Cu did not shown any effect on the pullulanase activity. The Pullulanase activity was almost recovered in the presence of Na ions, whereas 61% activity was recovered in the presence of K and (Table 4.6).

Table 4.6: Effect of various metals/reagents on pullulanase activity

Sr. No.	Metals	% Relative activity
1	Purified Pullulanase	100
2	EDTA	0
3	$MgSO_4$	47 ± 1.56
4	$CaCl_2$	42 ± 1.77
5	$FeSO_4$	0
6	$CuCl_2$	0
7	$ZnSO_4$	42 ± 1.80
8	KCL	61 ± 1.48
9	NaCl	92 ± 1.56

4.4 Discussion

The starch/sugar industry predominantly rely on pullulanase, an important debranching enzyme, in order to hydrolyse the α-1, 6 glucosidic linkages in starch. Therefore, pullulanase in conjunction with other enzymes that break down starch has been widely used in starch/sugar industry (Henrissat, 1991). Screening new bacterial strains or improving bacterial strains for the maximal production of pullulanase is a prerequisite for an industrial applications (Kłosowski et al., 2010; Domań-Pytka and Bardowski, 2004). Among the pullulanase that have been cloned and characterized from

many bacteria, most of them are type II pullulanase. Very few type I pullulanase are characterized and their enzymatic properties are explored (Yang et al., 2017; Liu et al., 2016; Wei et al., 2015, 2014; Qiao et al., 2015; Li et al., 2012; Kang et al., 2011; Bertoldo et al., 1999)

As *Microbulbifer* sp. predominantly degrade multiple polysaccharides. They have been attracted the focus of researchers paving the way for genomes sequencing and annotation in order to identify polysaccharide-degrading genes from potential *Microbulbifer* sp. Besides, the genome of *Microbulbifer mangrovi* DD-13^T, genome of seven *Microbulbifer* sp. have been sequenced. However pullulanase gene have reportedly been identified in the genome of *Microbulbifer agarilyticus* (WP_077400547.1) and *Microbulbifer* sp. CCB-MM1 only (Moh et al., 2017). In present study, based on the sequence analysis pullulanase gene identified in *Microbulbifer mangrovi* DD-13^T genome is classified as type I pullulanase (Detail analysis described in chapter 5). To the best of our knowledge none of the pullulanase genes have been cloned from *Microbulbifer* sp. nor their biochemical properties been studied. Present study is the first report on cloning of the pullulanase encoding gene of *Microbulbifer* sp. and its expression in *E.coli*.

The pET series vectors have been frequently used for the expression of pullulanase in *E.coli* Bl-21 (DE3) (Li et al., 2012; Liu et al., 2016; Wei at al., 2015; Kang et al., 2011). In order to achieve the expression of foreign genes, in-frame ligation of insert is prerequisite. Furthermore, addition of restriction sites for the appropriate restriction enzymes in PCR primer is crucial to facilitate the in-frame cloning of foreign genes in selected expression vectors. *In-silico* analysis of pullulanase gene sequence of *Microbulbifer mangrovi* DD-13^T suggest the absence of restriction site for *Hind*III and *Xho*I that in turn seem to be the criteria for introducing the restriction sites for these two

enzymes in the PCR primers. Furthermore, the *Hind*III and *Xho*I ends in the PCR product facilitated their ligation in-frame of pelB of pET-22b.

Purification of His-tagged proteins have reportedly been achieved using Ni-NTA agarose column (Qiao et al., 2015; Wei et al., 2014; Liu et al., 2016; Wei et al., 2015; Kang et al., 2011). Similarly in present study, using the Ni-NTA agarose column, pullulanase was 6.54 fold purified from cell free sonicated extract of BL21-pET22b-Pull. The purification fold achieved in present study was more than the purification fold reportedly achieved in *Paenibacillus polymyxa* Nws-pp2 (4.28 fold), *Paenibacillus barengoltzii* (3.6 fold), *Bacillus megaterium* (1.3 fold) (Wei et al., 2015; Liu et al., 2016; Yang et al., 2017) whereas less than the *Bacillus cerus* Nws-bc5 (9.3 fold), *Bacillus* sp. CICIM 263 (52.1 fold) (Wei et al., 2014; Li et al., 2012). Similarly, the specific activity of purified recombinant pullulanase was achieved to be 30.7 U/mg which is significantly higher than the specific activity of type-I pullulanase reported earlier from *Paenibacillus polymyxa* Nws-pp2 (16.17 U/mg), *Exigubacterium acetylicum* (22.1 U/mg) and *Thermotoga neapolitana* (28.7 U/mg) (Wei et al., 2015; Qiao et al., 2015; Kang et al., 2011). However, recombinant type-I pullulanase reported from *Bacillus* sp. CICIM 263, *Bacillus cerus* Nws-bc5, *Bacillus megaterium* WW1210 and *Paenibacillus barengoltzii* had higher specific activity than the recombinant pullulanase of *Microbulbifer mangrovi* DD-13^{T} (Li et al., 2012; Wei et al., 2014; Yang et al., 2017; Liu et al., 2016).

The product of pullulanase gene of *Microbulbifer mangrovi* DD-13^{T} depicted the molecular mass of approx. 85 KDa on SDS PAGE which is closer to the apparent molecular weight (90 KDa) predicted using bioinformatics tool. The molecular weight of pullulanase from *Microbulbifer mangrovi* DD-13^{T} was closer to the molecular weight of type-I pullulanase from *Thermotoga neapolitana* (93 kDa) whereas comparable to *Paenibacillus polymyxa* Nws-pp2 (96 KDa) (Kang et al. 2011; Wei et al., 2015). On the

contrary, the molecular weight of the recombinant pullulanase from *Microbulbifer mangrovi* DD-13^{T} is remarkably differrent from type-I pullulanase cloned from *Bacillus megaterium* WW1210 (112 KDa), *Bacillus* sp. CICIM 263 (100 KDa), *Exigubacterium acetylicum* (100 KDa) *Paenibacillus barengoltzii* (75 KDa) and *Bacillus cerus* Nws-bc5 (81 KDa) (Yang et al., 2017; Li et al., 2012; Qiao et al., 2015; Liu et al., 2016; Wei et al., 2014).

Additionally, unlike the type I pullulanase reported earlier, 85 KDa pullulanase cloned from *Microbulbifer mangrovi* DD-13^{T} showed optimal activity at 45 ℃ (Table 4.7). Type-I pullulanase reported from *Paenibacillus polymyxa* Nws-pp2 and *Bacillus cerus* Nws-bc5 are the only cold adapted type-I pullulanase that showed optimal activity at 35℃ and 40℃ respectively (Wei et al., 2015; 2014). The temperature reported for the optimal activity of type-I pullulanase from *Bacillus megaterium* WW1210, *Paenibacillus barengoltzii*, *Exigubacterium acetylicum*, *Bacillus* sp. CICIM 263 and *Thermotoga neoplitana* is ranging from 50 ℃ – 85 ℃, that is different from the assay temperature observed for the optimal activity of the recombinant pullulanase from *Microbulbifer mangrovi* DD-13^{T} (Table 4.7). Additionally, the purified pullulanase cloned from DD-13^{T} is partially active at 25℃ to 40℃. Similar thermal stabilities were reported for the pullulanase cloned from *Paenibacillus barengoltzii*, *Bacillus cerus* Nws-bc5, *Bacillus megaterium* WW1210, *Paenibacillus polymyxa* Nws-pp2 (Wei et al., 2014; Yang et al., 2017; Wei et al., 2015; Liu et al., 2016). On the contrary, *Thermotoga neapolitana* (fully active at 75℃ for 4 h), *Bacillus* sp. CICIM 263 (stable at 70℃ for 1 h) and *Exigobacterium acetylicum* (stable at 50℃ for 10 min) demonstrated thermal stabilities in comparison to the thermal stability of pullulanase from *Microbulbifer mangrovi* DD-13^{T} (Kang et al., 2011; Li et al., 2012; Qiao et al., 2015).

Table 4.7: Comparison of biochemical properties of type-I pullulanase cloned from *Microbulbifer mangrovi* DD-13^T with other reported type-I pullulanase

S. No.	Pullulanase cloned from various bacteria	Molecular mass (KDa)	pH	Temperature (℃)	References
1	*Microbulbifer mangrovi* DD-13T	85	7.0	45	Present study
2	*Bacillus megaterium* WW1210	112	6.5	55	Yang et al., 2017
3	*Paenibacillus barengoltzii*	75	5.5	50	Liu et al., 2016
4	*Paenibacillus polymyxa* Nws-pp2	99	6.0	35	Wei et al., 2015
5	Exigubacterium acetylicum	100	6.0	50	Qiao et al., 2015
6	*Bacillus cerus* Nws-bc5	80	6.0	40	Wei et al., 2014
7	*Bacillus* sp. CICIM 263	101	Alkaline & Neutral	70	Li et al., 2012
8	*Thermotoga neoplitana*	96	6.5	80-85	Kang et al., 2011

Like type I pullulanase cloned from *Bacillus* sp. CICIM263, recombinant pullulanase from DD-13^T is optimally active at neutral pH, whereas it differs from the type I pullulanase reported from *Bacillus megaterium* WW1210, *Paenibacillus barengoltzii*, *Paenibacillus polymyxa* Nws-pp2, *Exigubacterium acetylicum*, *Bacillus cerus* Nws-bc5 and *Thermotoga neoplitana* that are optimally active at slightly acidic pH

(Li et al., 2012; Yang et al., 2017; Liu et al., 2016; Wei et al., 2015; Qiao et al., 2015; Wei et al., 2014; Kang et al., 2011). Recombinant pullulanase cloned from *Microbulbifer mangrovi* DD-13^T remains stable in the pH range of 5.0 – 7.0 and most stable at pH 7.0 for 1 h. Similarly, type-I pullulanase cloned from *Bacillus* sp. CICIM 263 are mostly stable at pH 7.0 (Li et al., 2012). However, in comparison to the pH stability period in present study, the type-I pullulanase reported earlier from *Exigubacterium acetylicum* depicted stability in the pH range of 5.0 – 9.0 for 10 min whereas pullulanase cloned from *Paenibacillus barengoltzii* remains stable from pH 5.5 – 10.5 for 30 min (Qiao et al., 2015; Liu et al., 2016). Similarly, Type –I pullulanase from *Bacillus megaterium* WW1210 is stable at pH 6.5 – 8.5 for 30 min (Yang et al., 2017). Moreover, the type – I pullulanase cloned from *Paenibacillus polymyxa* Nws-pp2 showed stability for 90 min in the pH range 5.5 – 8.5 (Wei et al., 2015)

Sequence Analysis of the Polysaccharide-Degrading Genes from *Microbulbifer mangrovi* DD-13^{T}

As mentioned in the chapter 3 and 4, several multiple polysaccharide-degrading genes detected in the genome of *Microbulbifer mangrovi* DD-13^T. Amongst several genes one of the pullulanase gene is cloned and expressed in *E.coli*. The sequence of cloned pullulanase gene as well as the other polysaccharide-degrading genes from strain DD-13^T were analysed by various bioinformatics tools. The present chapter describes the methodology employed for the analysis of the gene sequences and the results obtained during analysis. Additionally, the genes annotated for polysaccharide-degradation were validated by experimental data. Furthermore, extensive discussion of the results obtained during present study is compared with reported literature to highlight the novelty of present study.

5.1 Materials

Agarose (Low melting point; LMP), alginate (Sodium salts), carrageenan (from Irish moss), xylan (from oat spelts), sodium salt of carboxymethyl cellulose (CMC), pectin, pullulan (extra pure), and starch (soluble) purchased from HiMEDIA, Mumbai, India. The other reagents used were of analytical grade.

Seaweeds (*Sargassum tenerrimum* and *Gracilaria corticata*) used in the present study were collected from the inter-tidal region of the Anjuna coast, Goa, India (N15°35′065″, E73°49′182″), in a sterile disposable polyethylene bags. The thalli were immediately washed several times with filtered seawater to remove epiphytes and later air dried overnight at 50 °C.

5.2 Methodology

5.2.1 Analysis of recombinant pullulanase gene sequence

Total number of nucleotide in pullulanase gene was determined. The nucleotide sequence was translated into amino acid sequence using ExPASy translate tool (http://web.expasy.org/translate/). The percentage G + C content was determined using GC calculator (http://www.endmemo.com/bio/gc.php). The molecular weight and pI of pullulanase was predicted using ExPASy- Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Furthermore, the pullulanase gene sequence was scanned for the presence of probable signal peptide using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The promoter sequence was determined using PePPer server (http://pepper.molgenrug.nl/index.php/prokaryote-promoters). The amino acid sequence of pullulanase in strain DD-13^T was compared against the conserved domain database (Marchler-Bauer et al., 2014) present at the NCBI's server to determine conserved domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

5.2.2 BLAST analysis of pullulanase gene

The nucleotide and amino acid sequence of pullulanase gene was subjected to BLASTx and BLASTp respectively and searched NCBI's nr- protein database to determine homologous sequences (https://blast.ncbi.nlm.nih.gov). The amino acid sequence of the pullulanase from the NCBI's nr-protein database that depicted significant similarity with pullulanase of *Microbulbifer mangrovi* DD-13^T with an e-value = 0, and demonstrate 99 % query cover was downloaded and used for multiple sequence alignment using Clustal W program. The signatory conserved region I- IV of the pullulanase gene was manually analysed. Phylogeny and molecular evolutionary analysis were conducted using MEGA (version 7) (Kumar et al., 2016).

5.2.3 Detection of CAZy domain in pullulanase gene

The pullulanase gene sequence was annotated using dbCAN (http://csbl.bmb.uga.edu/dbCAN/annotate.php). dbCAN is a database for automated Carbohydrate active enzyme annotation. Briefly the amino acid sequence of the pullulanase was uploaded in dbCAN server. The co-ordinate for the CAZy domain was noted from the output section of the server. Furthermore, based on the coordinate information for CAZy domain, the map for pullulanase gene was constructed.

5.2.4 Analysis of other polysaccharide-degrading genes sequence identified through annotation of genome of *Microbulbifer mangrovi* DD-13^T

As mentioned in chapter 3, the genome annotation led to identification of several polysaccharide-degrading genes such as agarase, alginate lyase, carrageenase, chitinase, amylase, pectinase, xylanase, cellobiase etc. The sequence of polysaccharide-degrading genes identified in the genome of *Microbulbifer mangrovi* DD-13^T were analysed as below-

5.2.4.1 General Characteristics of polysaccharide-degrading genes of *Microbulbifer mangrovi* DD-13^T

Total number of nucleotide in polysaccharide-degrading genes were determined. The percentage G + C content was determined using GC calculator (http://www.endmemo.com/bio/gc.php). The nucleotide sequence of polysaccharide-degrading genes was translated in to amino acid sequence using ExPASy translate tool (http://web.expasy.org/translate/). The molecular weight and pI of polysaccharide-degrading genes were predicted using ExPASy- Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Furthermore, the amino acid sequence of polysaccharide-degrading genes were analysed for the presence of probable signal

peptide using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The promoter sequence was determined using PePPer server (http://pepper.molgenrug.nl/index.php/prokaryote-promoters). The amino acid sequence of polysaccharide-degrading enzymes were analysed for conserved domain against the conserved domain database (Marchler-Bauer et al., 2014) using NCBI's server for conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

5.2.4.2 BLAST and evolutionary analysis of polysaccharide-degrading genes from *Microbulbifer mangrovi* DD-13^T

The similarity search of the polysaccharide-degrading genes of *Microbulbifer mangrovi* DD-13^T against the NCBI's nr-protein database was done using BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The homologous sequence of nr-database depicting significant similarity to the polysaccharide-degrading genes of strain DD-13^T were downloaded and used as input sequence for the multiple sequence alignment using the Clustal W programme. Furthermore, the phylogenetic and evolutionary analysis of the polysaccharide-degrading genes of bacterial strain DD-13^T was done using the MEGA (version 7) software.

5.2.4.3 Homology modelling of polysaccharide-degrading proteins of *Microbulbifer mangrovi* DD-13^T using SWISS Model

The homology model for the polysaccharide-degrading enzymes of *Microbulbifer mangrovi* DD-13^T was made using SWISS MODEL (https://swissmodel.expasy.org/). The homology model building involves three steps *viz*. template search against the target sequence, template selection and model building. Briefly, The SWISS MODEL building sever search the target sequence with BLAST (Altschul et al., 1997) against the primary

amino acid sequence contained in the SMTL (SWISS MODEL template library). For each identified template, the template's quality has been predicted from features of the target-template alignment. The templates with the highest quality have then been selected for model building. Server utilizes ProMod3 to build the models based on the target-template alignment.

5.2.4.4 Detecting the modules in polysaccharide-degrading enzymes from *Microbulbifer mangrovi* DD-13^T

The polysaccharide-degrading gene of *Microbulbifer mangrovi* DD-13^T was analysed for the presence of CAZymes domain. Briefly, the amino acid sequence of the polysaccharide-degrading genes was uploaded on the dbCAN (http://csbl.bmb.uga.edu/dbCAN/annotate.php) server. The co-ordinate obtained for the CAZymes domain of the polysaccharide-degrading genes was noted from the output section of the server. Furthermore, based on the coordinate information for CAZymes domain, the map of polysaccharide-degrading genes containing multiple CAZymes domain was constructed.

5.2.4.5 Identification and distribution of CAZymes in the genome of *Microbulbifer mangrovi* DD-13^T

As mentioned in chapter 3, total 3749 genes were predicted in the genome of *Microbulbifer mangrovi* DD-13^T using Glimmer. These genes were analyzed against CAZy database (Lombard et al. 2014) for being a probable CAZyme. Briefly, the sequence file of the predicted gene was uploaded in a CAZyme Analysis Toolkit (CAT) server (Park et al. 2010). The threshold e-value was kept $1e^{-5}$. Information on the pfam domain with a default bit score of 55 was included.

5.2.5 Validation of genomic data

Genome annotation of *Microbulbifer mangrovi* DD-13^T revealed the presence of several polysaccharide-degrading genes in the genome, indicating the extra ordinary potential of strain DD-13^T to degrade multiple. To validate the genomic potential of strain DD-13^T for polysaccharide-degradation, following experiments were performed:-

5.2.5.1 Agar and alginate degradation by *Microbulbifer mangrovi* DD-13^T

The starter culture was prepared by inoculating a loopful of strain DD-13^T culture in an artificial seawater (ASW) medium (g L^{-1}; Tris base 6.05, MgSO4·7H2O 12.32, KCL 0.74, (NH4)2HPO4 0.13, NaCl 17.52, and CaCl2 0.14) containing 0.2% of alginate or 0.2% low melting point agarose and incubated at 30 °C for 24 h on an orbital shaker at 150 rpm. One ml of the starter culture was inoculated into 100 mL of ASW containing 0.2% sodium alginate/agarose and grown at 30 °C on an orbital shaker at 150 rpm. At an interval of 8 h, 3 mL of culture was aseptically collected to determine the OD_{600}. The remaining fraction was centrifuged at 4 °C and 10,000 rpm for 10 min, and the culture supernatant was collected. The reducing sugar in the culture supernatant was determined by the 3,5′-dinitrosalicylic acid (DNSA) method (Miller 1959). Briefly, 0.5 mL of DNSA reagent was added to 0.5 mL of culture supernatant. The reaction tubes were incubated in a boiling water bath for 10 min. Reaction tubes were cooled, and optical density (OD) was determined at 540 nm. Galactose was used as standard. The agarase activity in the culture supernatant was determined at 50 °C by the DNSA method with galactose as reference sugar (Miller 1959).

The alginate lyase activity in the culture supernatant was determined at 50 °C using maltose as reference sugar (Nelson 1944). The assay mixture contained 0.5 mL sodium alginate (0.1%, w/v prepared in 20 mM phosphate buffer) and 0.2 mL of culture

supernatant (enzyme). The reaction was carried out for 60 min at 50 °C. Nelson reagent A (0.7 mL) was added, and tubes were kept in a boiling water bath for 15 min followed by cooling at 0 °C. Nelson reagent B (0.35 mL) was added. The reaction mixture was mixed and incubated for 5 min at 30 °C. Absorbance was measured at 680 nm using a spectrophotometer (Shimadzu Co. Kyoto, Japan). Appropriate substrate and enzyme blanks were included. The final OD was calculated after subtracting the OD of substrate and enzyme blank. One unit of alginate lyase was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute at 50 °C.

5.2.5.2 Seaweed degradation by *Microbulbifer mangrovi* DD-13^T

Sargassum tenerrimum and *Gracilaria corticata* were collected from the inter-tidal region of Anjuna coast, Goa, India (N15°35′065″, E73°49′182″), in sterile disposable polyethylene bags. The thalli were washed thoroughly with filtered seawater to remove epiphytes and later air dried overnight at 50 °C. The dried thalli were grinded in a 600-W blender to obtain fine powder that was stored at 4 °C in a screw-capped vials.

One mL of 24-h grown starter culture was inoculated in 100 mL of ASW supplemented with 0.1% (w/v) of *Sargassum* or *Gracilaria* powder and grown at 30 °C on an orbital shaker (150 rpm). At an interval of 24 h, 3 mL of culture was aseptically collected and OD_{600} was determined. Furthermore, the remaining fraction was centrifuged and the culture supernatant was recovered. The reducing sugar in the culture supernatant was determined as described previously. Additionally, agarase or alginate lyase activity in the culture supernatant was also determined. ASW medium with 0.1% seaweed powder without any bacterial inoculum was used as control.

5.2.5.3 Bacterial growth studies with other polysaccharides and determination of homologous carbohydrase activities

The bacterial strain DD-13^T was inoculated in ASW medium containing 0.2% one of the following polysaccharides as carbon source: carrageenan (from Irish moss), xylan (from oat spelts), sodium salt of carboxymethyl cellulose (CMC), pectin, pullulan (extra pure), and starch (soluble). After growing the culture for 24 h on an orbital shaker (150 rpm) at 30 °C, optical density was immediately determined at 600 nm using a spectrophotometer (Shimadzu Co. Kyoto, Japan). Additionally, the homologous carbohydrase activity from the 24-h cell-free culture supernatant was spectrophotometrically determined by the DNSA method (Miller 1959) using 0.2% of the respective polysaccharide that was used as growth substrate. Pectin and CMC were resuspended in 0.1 M citrate buffer (pH 5) for determination of pectinase and CMCase activities, respectively, whereas carrageenan, pullulan, and xylan were resuspended in 20 mM TrisCl (pH 7) for detection of carrageenase, pullunase, and xylanase activities. All the carbohydrase activities were determined at 37 °C whereas CMCase activity was determined at 45 °C. Glucose was used as reference sugar. Appropriate enzyme and substrate blanks were included in the assay. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute at respective temperatures.

5.3 Results

5.3.1 Characteristics of pullulanase gene from *Microbulbifer mangrovi* DD-13^T

As described in chapter 4, the pullulanase encoding gene of *Microbulbifer mangrovi* DD-13^T is cloned in pET-22b and expressed in *E.coli* Bl-21 (DE3). The coding region of pullulanase gene cloned in pET-22b and the translated amino acids along with ATG and TAA identified as start and stop codon in the ORF of the pullulanase gene (Fig 5.1). The start and stop codons are highlighted in red (Fig 5.1). Cloning of the pullulanase gene in pET-22b in proper orientation was confirmed by resequencing of the cloned DNA fragment that encodes pullulanase. The *in-silico* analysis for cloning of the obtained sequence confirms the pullulanase gene cloned in pET-22b in proper orientation. The sequence analysis indicates that the coding region of pullulanase gene of *Microbulbifer mangrovi* DD-13^T is a stretch of 2415 bp with G+C content of 54.20 %. The pullulanase gene of *Microbulbifer mangrovi* DD-13^T encodes a protein of 804 amino acids. The molecular weight and pI of pullulanase is predicted to be 90 KDa and 4.6 respectively (Table 5.1). Furthermore, the scanning of amino acid sequence of pullulanase gene by Signal P detected a signal peptide of 29 amino acids at the N-terminal of the pullulanase. The amino acid sequence of the signal peptide comprises MIKPTHGGRLRLPAVPCLLLAAFSTSVSA. The cleavage site of signal peptide is predicted between Ala^{29} and Glu^{30} residues of pullulanase of *Microbulbifer mangrovi* DD-13^T. To keep the expression of cloned pullulanase gene under the control of promoter of the expression vector and secretion of recombinant pullulanase under the control of pelb, the predicted DNA coding signal peptide was excluded during the cloning of pullulanase gene by designing appropriate primers as described previously.

```
atgacccaggttagccagaacgcatttcacacctgccagtcgttcgccaacggcgatagc
 M  T  Q  V  S  Q  N  A  F  H  T  C  Q  S  F  A  N  G  D  S
agcggcggcccccgcttcaaggtggatcgtcacggcgactggagcgaaagctatcccact
 S  G  G  P  R  F  K  V  D  R  H  G  D  W  S  E  S  Y  P  T
gcagattacgccgtcaccggcaaccgctcctacgaaatcacctttcaaagcgacagcctc
 A  D  Y  A  V  T  G  N  R  S  Y  E  I  T  F  Q  S  D  S  L
aacatcgctgtcaatgaagtggcgagctgcgatgaaggcagcgtccatggcgccaatttt
 N  I  A  V  N  E  V  A  S  C  D  E  G  S  V  H  G  A  N  F
tcctcactctattttcgcggaacacccaacacatggaacgcctcttccatgaatctggtg
 S  S  L  Y  F  R  G  T  P  N  T  W  N  A  S  S  M  N  L  V
gccgacaatacctggcaactgcagatccactttgacggccaggcaaaccagcggttcaag
 A  D  N  T  W  Q  L  Q  I  H  F  D  G  Q  A  N  Q  R  F  K
ctcgatgtgttcggtgactggagccagaattacggtgataacaacagcgacggcatcctg
 L  D  V  F  G  D  W  S  Q  N  Y  G  D  N  N  S  D  G  I  L
gatcaaaccggtagtgatatttatacggacgtggtcggcgattacctgctaacggtcaac
 D  Q  T  G  S  D  I  Y  T  D  V  V  G  D  Y  L  L  T  V  N
gatgcgaacctcaactacactctggaaccggttgaatgcaccgaaaactgcaatcaaagc
 D  A  N  L  N  Y  T  L  E  P  V  E  C  T  E  N  C  N  Q  S
ctggataccctgggtgcagtgtactcctcaacggaaaccactttctccctgtggtctcca
 L  D  T  L  G  A  V  Y  S  S  T  E  T  T  F  S  L  W  S  P
gatcacaacgacgtacaactggttctcgatggccagaattatccgatggaaaagattccc
 D  H  N  D  V  Q  L  V  L  D  G  Q  N  Y  P  M  E  K  I  P
gacgctgcgcttgccgcacaggggatgacagacatctattcggttaccgtaagcggcgac
 D  A  A  L  A  A  Q  G  M  T  D  I  Y  S  V  T  V  S  G  D
tggaaactgaagccctattattttgtcgtcaatggcgtagtggtccgcgatccttacggc
 W  K  L  K  P  Y  Y  F  V  V  N  G  V  V  V  R  D  P  Y  G
aagatggtggaacccgatagcgataacaatatcgtcatggatatgtccgctacagagtta
 K  M  V  E  P  D  S  D  N  N  I  V  M  D  M  S  A  T  E  L
cccaacggatggtccgcacgccctttactcaatgagcgtgaggacgcggtaatctacgag
 P  N  G  W  S  A  R  P  L  L  N  E  R  E  D  A  V  I  Y  E
gtgcacgtacgcgatttcactattgcacccgagtccggtgtcagcagtgacaagcgcgga
 V  H  V  R  D  F  T  I  A  P  E  S  G  V  S  S  D  K  R  G
aaatttctcggcatggtcgaaagcggtagccgtttcaatggcgtggcgacgggtatcgac
 K  F  L  G  M  V  E  S  G  S  R  F  N  G  V  A  T  G  I  D
cacctgaaggaactcggtgtgacgcatgtgcaattgctacccgtgtatgacttcaatgcc
 H  L  K  E  L  G  V  T  H  V  Q  L  L  P  V  Y  D  F  N  A
tgcgccgaccctgccgacactgcctgttacagctggggttacgacccgcgcaatttcaac
 C  A  D  P  A  D  T  A  C  Y  S  W  G  Y  D  P  R  N  F  N
gtaccggaagaacgctactcgcagacacccacggactacgaaaatcgcgtaagcgaattc
 V  P  E  E  R  Y  S  Q  T  P  T  D  Y  E  N  R  V  S  E  F
aagaccatggtcgacgagtttcacaaagccggtattcgagtcattatggatgtggtctac
 K  T  M  V  D  E  F  H  K  A  G  I  R  V  I  M  D  V  V  Y
aaccacacttattcgaaatctgttttcgagccaatatccagcagctactacaccccaacc
 N  H  T  Y  S  K  S  V  F  E  P  I  S  S  S  Y  Y  T  P  T
gacctgtccggcactggcaacagtattgatgccaatgtccccatggtcggccgcatgatc
 D  L  S  G  T  G  N  S  I  D  A  N  V  P  M  V  G  R  M  I
cgcgactccctagaatattgggtagatgaatacaatattgacggtttcgttttgacctg
 R  D  S  L  E  Y  W  V  D  E  Y  N  I  D  G  F  R  F  D  L
atcggtatttttgactatgacgaagtggccgattgggcggacaacctgaatagcaaat
 I  G  I  F  D  Y  D  E  V  A  D  W  A  D  N  L  N  S  K  F
ccggaccgcaacctgttgatctacggcgaaccctggaacggctttgcggcagactcgcgc
 P  D  R  N  L  L  I  Y  G  E  P  W  N  G  F  A  A  D  S  R
cagccggagcgcgtgcgcttgggcaccatcggccgtatacacgagtctcacgccggggtg
 Q  P  E  R  V  R  L  G  T  I  G  R  I  H  E  S  H  A  G  V
ttcaacccgaaatttcgtgaagccatcaaaggacaaaacgataacggcggctgcaacccc
 F  N  P  K  F  R  E  A  I  K  G  Q  N  D  N  G  G  C  N  P
ggcgactgcttcgctctcaataacaatcccgatacctggcgaatcgaagtgggcagccgc
 G  D  C  F  A  L  N  N  N  P  D  T  W  R  I  E  V  G  S  R
ggcggtctccgctacacaaaagacaaggatacggccatcgacacctgggacccgatgttt
 G  G  L  R  Y  T  K  D  K  D  T  A  I  D  T  W  D  P  M  F
gcgatggatccggaacaaagcatcaactacgtctcggcccacgataacctgaccttgcgg
 A  M  D  P  E  Q  S  I  N  Y  V  S  A  H  D  N  L  T  L  R
gacaagatcctgcaatgggcagacttgaatggcatcagcagggatagcggctacttgcga
 D  K  I  L  Q  W  A  D  L  N  G  I  S  R  D  S  G  Y  L  R
agaatccagatgttcgccaacgggattgttctaaccagccagggtattccattcctgcac
 R  I  Q  M  F  A  N  G  I  V  L  T  S  Q  G  I  P  F  L  H
ggcggtgtcgaactcatgcgcgacaagcaggaggatcacaacagctacgattcgccggac
 G  G  V  E  L  M  R  D  K  Q  E  D  H  N  S  Y  D  S  P  D
gcaatcaaccagtattactggcagtggaaaatcgacaatgctgatatctacgcgtactac
 A  I  N  Q  Y  Y  W  Q  W  K  I  D  N  A  D  I  Y  A  Y  Y
aaagatgtgattgcactacgccgcgcgcaccccgctttccggcttaccagctgggatgca
 K  D  V  I  A  L  R  R  A  H  P  A  F  R  L  T  S  W  D  A
atcgaccagcacatgaccagcaaccggccacgctacggtattgtagtccatcacattgat
 I  D  Q  H  M  T  S  N  R  P  R  Y  G  I  V  V  H  H  I  D
ggcgctgcggtaggtgacagctggagtgaagttattgtgatctacaacagtgcggacaat
 G  A  A  V  G  D  S  W  S  E  V  I  V  I  Y  N  S  A  D  N
tacacctacagcttgcccgcgggcgagtggaaagtggcactggaaaaatcagaccccgct
 Y  T  Y  S  L  P  A  G  E  W  K  V  A  L  E  K  S  D  P  A
gcgggtaacggacgcagcgtgagtggctccgtggtggcagaggggaccgcggttaccgtg
 A  G  N  G  R  S  V  S  G  S  V  V  A  E  G  T  A  V  T  V
ctttaccgccattaa
 L  Y  R  H  -
```

Fig 5.1: Nucleotides/amino acids constituting the full coding region of pullulanase gene in *Microbulbifer mangrovi* DD-13^T cloned in expression vector. Start and stop codon highlighted in red.Amino acid residues constituting CBM and GH domains are highlighted in grey and yellow respectively

Table 5.1: Characteristics of pullulanase gene from *Microbulbifer mangrovi* DD-13^T

Sr. No.	Protein name	Position of genes	ORF detail (Strand), frame	% GC content	No. of amino acids	Predicted Molecular weight (KDa)	Predicted pI
1	Pullulanase	contig1082: 12386-14932	+1	54.20	804	90	4.60

5.3.2 Homology analysis of pullulanase of *Microbulbifer mangrovi* DD-13^T

BlastP analysis indicates that the pullulanase gene of *Microbulbifer mangrovi* DD-13^T is 79% identical with the amino acid sequence of the pullulanase of *Microbulbifer agarilyticus* (Table 5.2). It is noteworthy that the pullulanase from *Microbulbifer agarilyticus* is the only pullulanase that demonstrate maximum identity (79%) to the DD-13^T pullulanase. However with the other non-Microbulbifer the pullulanase from *Microbulbifer mangrovi* DD-13^T demonstrated ≤ 55% identity. As observed from table 5.2 only 55 % identity was observed with the pullulanase of *Photobacterium halotolerans* and *Pseudoalteromonas denitrificans* (55%). The range of identity of pullulanase with pullulanase of other bacteria ranged from 49 – 55 %. It is also very evident from the Table 5.2, that the pullulanase of DD-13^T depicts similarity with the type I pullulanase of type-species of diverse bacterial genus including *Pseudoalteromonas, Pseudobacteriovorax, Orenia, Halonatronum, Anaerobranca, Bacillus, Kosmotoga, Thermobacillus, Penibacillus* and *Thermoanaerobacter* indicating that the pullulanase of DD-13^T is probably a type I pullulanase. It is noteworthy that the pullulanase of DD-13^T depicts low similarity (34% - 47 %) with the reported type I pullulanase of the NCBI's nr-protein database with low e-value and high query cover which suggest that the DD-13^T pullulanase is a novel type I pullulanase. The NCBI conserved domain search indicates the presence of AmyAc_Pullulanase_LD-like domain (alpha amylase catalytic domain found in pullulanase) in the pullulanase of *Microbulbifer mangrovi* DD-13^T.

Table 5.2: Results of BLAST analysis with respect to amino acid sequence against pullulanase from *Microbulbifer mangrovi* DD-13^T from NCBI's nr-protein database.

Sr. No.	Bacterial pullulanase	Type of pullulanase	Identity (%) with strain DD-13 pullulanase	Query Cover (%)	E-value	Accession no.
1	*Microbulbifer agarilyticus*	-	79	99	0.0	WP_077400547.1
2	*Photobacterium halotolerans*	-	55	99	0.0	WP_027251440.1
3	*Pseudoalteromonas denitrificans*	Type I	55	100	0.0	WP_091988381.1
4	*Photobacterium galatheae*	-	54	99	0.0	WP_036748130.1
5	*Cellvibrio japonicus*	-	53	99	0.0	WP_012487762.1
6	*Psychromonas aquimarina*	-	53	99	0.0	WP_028862995.1
7	*Agarivorans gilvus*	-	50	99	0.0	WP_055733099.1
8	*Agarivorans albus*	-	49	99	0.0	WP_016401950.1
9	*Pseudobacteriovorax antillogorgiicola*	Type I	47	77	0.0	SMF28584.1
10	*Orenia marismortui*	Type I	40	77	4e-129	WP_018248060.1
11	*Orenia metallireducens*	Type I	38	77	3e-125	WP_068719816.1
12	*Halonatronum saccharophilum*	Type I	39	76	1e-124	WP_027338833.1
13	*Anaerobranca gottschalkii*	Type I	39	79	2e-119	AAS47565.1
14	*Bacillus thermoamylovorans*	Type I	39	76	2e-117	WP_052480346.1
15	*Anaerobranca californiensis*	Type I	38	81	2e-117	WP_084672393.1
16	*Bacillus hisashii*	Type I	39	76	8e-117	WP_095142450.1
17	*Kosmotoga arenicorallina*	Type I	38	77	3e-115	WP_068346132.1
18	*Bacillus gottheilii*	Type I	38	76	1e-114	WP_080843939.1
19	*Thermobacillus composti*	Type I	38	76	3e-113	WP_015255773.1
20	*Paenibacillus polysaccharolyticus*	Type I	36	78	3e-112	WP_090915449.1
21	*Paenibacillus amylolyticus*	Type I	34	76	3e-112	WP_076329145.1
22	*Paenibacillus assamensis*	Type I	38	77	3e-112	WP_036605074.1
23	*Thermoanaerobacter kivui*	Type I	37	73	5e-112	WP_049685052.1
24	*Parabacteroides distasonis*	Type I	37	77	6e-112	WP_057326458.1
25	*Bacillus korlensis*	Type I	38	75	6e-112	WP_084362145.1
26	*Paenibacillus polymyxa*	Type I	37	76	3e-111	WP_039272910.1

5.3.3 Multiple sequence alignment of conserved region in pullulanase of *Microbulbifer mangrovi* DD-13^{T} and other bacterial pullulanase from NCBI's nr-protein database

The amino acid sequence of pullulanase from NCBI's nr-protein database showing significant similarity (top hits) with the pullulanase of *Microbulbifer mangrovi* DD-13^{T} were considered as homologous sequence and subjected to the multiple sequence alignment with the pullulanase from strain DD-13^{T}. The multiple amino acid sequence alignment analysis reveals the presence of YSWGYDP conserved motif in the pullulanase of *Microbulbifer mangrovi* DD-13^{T} (Fig 5.2). The conserved region YNWGDP is reported to observed only in type-I pullulanase and not in any other class of pullulanase, confirming the classification of pullulanase in *Microbulbifer mangrovi* DD-13^{T} as type-I pullulanase. Furthermore, the multiple amino acid sequence alignment analysis enables the identification of highly conserved region I-IV in the pullulanase of *Microbulbifer mangrovi* DD-13^{T}. The conserved region I-IV are one of the fundamental characteristics of the pullulanase and has been observed in all the reported pullulanase. The conserved region I-IV identified in the pullulanase of *Microbulbifer mangrovi* DD-13^{T} are similar to those present in type I pullulanase and specifically involves in the hydrolysis of α-1,6- glycosidic linkage in pullulan, starch and amylopectin. The highly conserved regions (Region I: GIRVIMDVIMDVVYNH; Region II: DGFRFDLIG; Region III: YGEPWNG and Region IV:DPEQSINYVSAHDN) identified are highlighted in yellow colour (Fig 5.2). Additionally, the conserved regions contain the acidic residues constituting the catalytic triad Asp^{479}, Glu^{510} and Asp^{615} of pullulanase in *Microbulbifer mangrovi* DD-13^{T}. These amino acid residues of catalytic triad is shown in the homology model of pullulanase of strain DD-13^{T} (Fig 5.3 c).

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                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
WP_077400547      MIKPICGGRT RLPVLSCLFI TLFSAQASAE WYFRGTPNGW SADAMTAVSS
WP_027251440      ----MRTLPT LIVGAT---- -LISAPALAD WHFRGTPNQW NAAAMTQITT
WP_036748130      ----MRTLPT LVIGAS---- -LISAPALAD WHFRGTPNQW NAAQMTQIAA
WP_012487762      ----MHTVNY RLALASCLFI CSFSTCVQAD WFFRGTPNSW GTVALTQTAS
WP_028862995      ----MNKK-L LFAGMI---- -TASLTVQAQ WEFRGTANNW QSTALLYISG
WP_055733099      ----MKKLSI VLLGAS---- -LLSAHASAE WNFRGTPNQW LSTPLELVSG
M. mangrovi       ---------- ---------- ---------- ---------- ----MTQVSQ

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
WP_077400547      NQFETCQSFA SGDANGGPRF KVDRYGDWQE NYPTSDYGVA AGQSYTITFY
WP_027251440      THYQTCQSFL QGDATGGPRF KIDRYGDWQE SYPADDYTVA GDQSYRIDFY
WP_036748130      NHYQTCQTFQ QGDATGGARF KIDRYGDWQE SYPASDYTVA GDQSYRIDFY
WP_012487762      NLYETCQHFA GGDANGGPRF KIDRYGNWTE NYPSADYAVS ANTHYKITFN
WP_028862995      NLYQTCQTFT DPDS----RF KIDRYGDWSE SYPGTDYRVT GDKSYRINFY
WP_055733099      NQYQTCQSFG DNNP----RF KIDRYGDWNE AYPNADYTVA ANKSYDIRFF
M. mangrovi       NAFHTCQSFA NGDSSGGPRF KVDRHGDWSE SYPTADYAVT GNRSYEITFQ
                      *** *          ** * ** *** *   *  **        * *

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
WP_077400547      SDSTSIQVAE VASCD--DTP GGFQSN---- LPSLFFRGTP NGWGTSELTL
WP_027251440      TDSQTIITTP VTSCD----S QSLAQN---- FAALHFRGTP NQWAAEAMTL
WP_036748130      PDSHSIQTTQ VASCD----S QAFAQN---- FNALYFRGTA NNWAADAMAL
WP_012487762      SSTRAISAQA VNHCQ----D TGFSQV---- FPSLYFRGTP NSWGTTAMAL
WP_028862995      SDSHVIETTE VTDCSGDNGD NGDPADYAQN FTSLNFRGTA NAWASTAMQL
WP_055733099      SDSKTITATE VANCG--DIE IEPPED---- --SWYFRGTA NGWQATPMDS
M. mangrovi       SDSLNIAVNE VASCD--EGS -VHGAN---- FSSLYFRGTP NTWNASSMNL
                             *  *                       ****   * *

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
WP_077400547      VDDNTWQVQV NFDGQANQRF KLDVNGDWSR NYGDNGADGT LEQTGADIFT
WP_027251440      VDNNTWSLLV HFDGQTSQRF KFDLTGDWSQ NYGDNQNDGV LDASGDDIYT
WP_036748130      VGDNTWSRLI HFDGQANQRF KFDLTGDWSQ NYGDNQNDGV LDAAGGDIYT
WP_012487762      VANNTWELTV NFDGQANQRF KFDVSGNWAT NYGDNNSDNI LDQTGADIYQ
WP_028862995      AADNTWQLTV TLDGQDQQRF KFDLLGDWTQ NYGDSDADGS LDLGGSDIFT
WP_055733099      SDNITFCTTQ TFAN-DEPRF KIDHYGDWTE NYP------- ----QADVLV
M. mangrovi       VADNTWQLQI HFDGQANQRF KLDVFGDWSQ NYGDNNSDGI LDQTGSDIYT
                      *                ** * *  * *   **

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
WP_077400547      GVTGEYLLTV NDASLTYTLE SVA-CSNNCG -GEVQTLGAV YNAAETRFSL
WP_027251440      QINGEYLVTV NDQTLNYTLT PVTPCTSDCP --PLPTLGAQ YQPDKTTFAI
WP_036748130      NVSGDYVVTV NDQTLVYSLR AVNPCTADCA --VQPSLGAI YQPDKTTFAI
WP_012487762      TGTGQYKITV NDLTRHYTVE AMP-CTVNCP -AQVNRLGAV YSNTATTFSI
WP_028862995      SASGDYIIEV NDASLTYSLT PADGCSVDCA DSSSDTLGAA YSTDSTTFSI
WP_055733099      DANSSYDICF N-ADTKVVTT EKQQCVDDCP -ITPETLGAV YSPSATTFSL
M. mangrovi       DVVGDYLLTV NDANLNYTLE PVE-CTENCN -QSLDTLGAV YSSTETTFSL
                       *     *              *           ***   *    * *
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                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290        300
WP_077400547      WSPDHSDVQL VLDGQSYPMD KAPDS----N DLTDVYSVAV SGDWKLKPYY
WP_027251440      WSPDHSDVTV TVNGTAYPLG KVSDA----N GYTDIYQVDV SGDLHLAEYT
WP_036748130      WSPDHSNVTV TVNGTEYPLS KVSDF----N GYTDVYQTEV SGDLYLAEYT
WP_012487762      WSPETGNVAV KVNGVIHNLQ AVPDF----N GYSNVYQVSV EGDLHLAEYQ
WP_028862995      WSPQHSNVKV RVNGVEYTMN KVADF----A GYSDIYQVKV NGDLHLQEYT
WP_055733099      WSPENSNVSV EVDGVSYAMQ AVPDF----A GYTQVYQATV PGDLHLKPYT
M. mangrovi       WSPDHNDVQL VLDGQNYPME KIPDAALAAQ GMTDIYSVTV SGDWKLKPYY
                  ***    *      *          *            *   *  **  *  *

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350
WP_077400547      FVVNGNSVRD PYGRMVEPNT NN----NIVM DLDSTDLPGG WSARPVMNAR
WP_027251440      FKINGIPVRD PYGKMAKPGT GDSEAINIVM DMSRTAPDGG WAERPALVNR
WP_036748130      FQINGIPVRD PYGKMVKPGT GDSEAINIVM DMSRTRPAGG WAERPALVNR
WP_012487762      FLLDGVAVRD PYGVMVKAQT N----INIVM DLARTQVADG WATRPALVAR
WP_028862995      YLINGIQVRD PYGKMAKPGT GDYEAVNIVM DMSRTEPAGG WATRPALIER
WP_055733099      FYVNGVQVRD PYGKMAQAGT GDYEAVNIVM DMSRTEPLDG WAARPSLIER
M. mangrovi       FVVNGVVVRD PYGKMVEPDS DN----NIVM DMSATELPNG WSARPLLNER
                      *  *** *** *            **** *   *    * *  **    *

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
WP_077400547      EDAIIYEVHV RDFTIASESG VSSNKRGKFL GMVESGTTYN GVATGIDHLK
WP_027251440      EDAVIYEVHV RDFTIDASAG VSADKRGKYL GMVESGTRYN GLKTGIDHLV
WP_036748130      EDAVIYEVHV RDFTIDASSG VSAAKRGKFL GMVESGTRYN GLKTGIDHLV
WP_012487762      EDAIIYEVHV RDFSIDTSSG VSAGKRGKYL GMVEQGTTYN GVKTGIDHLK
WP_028862995      EDAVIYEAHV RDFTIDSSSG VSAGNHGKFM GMVETGTRYN GVKTGIDHLK
WP_055733099      EDAIIYELHV RDFTIDASSG VSADKRGKFL GLVEPGTTYQ GITTGIDHLK
M. mangrovi       EDAVIYEVHV RDFTIAPESG VSSDKRGKFL GMVESGSRFN GVATGIDHLK
                  ******* ** *****    * **  * **   * ** *     *  ******

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
WP_077400547      ELGVTHVQLL PSYDFGSCPD VTDN-SCYNW GYDPRNYSIP EERYSLTPFD
WP_027251440      DLGITHVQLL PVYDFATCDG LPDSDPCYNW GYDPRNYNVP EERYSQVPTD
WP_036748130      DLGVTHVQLL PVFDFATCDG LPDSDPCYNW GYDPRNYNVP EERYSQVPTD
WP_012487762      ELGVTHVQLL PVYDFATCDG LPDNDPCYNW GYDPRNYNVP EERYSQTPLD
WP_028862995      ELGVTHVQLL PVYDFATCDG LPDSNSCYNW GYDPRNFNIP EDRYSKVPTD
WP_055733099      ELGVTHVQLL PVYDFATCDG LPDSDPCYNW GYDPRNYNIP EDRYSNVPND
M. mangrovi       ELGVTHVQLL PVYDFNACAD PADT-ACYSW GYDPRNFNVP EERYSQTPTD
                   ** ****** *  **  *     *   ** * ****** * * * ***  * *

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
WP_077400547      YENRVREFKQ MVDEFHKAGI RVIMDVVYNH TYAKEMFEPI STSYYTPTDL
WP_027251440      YEARASEFKT MVNEFHKAGI RVIMDVVYNH TYAKEMFENI SNSYYTPTDL
WP_036748130      YEARANEFKT MVNEFHKAGI RVIMDVVYNH TYANEMFENI SNRYYTPTDL
WP_012487762      YENRVREFKT MVNEFHKAGI RVIMDVVYNH TYSKDMFEAI SPRYYTPTDL
WP_028862995      YEERAREFKT MVNEFHKAGI RVIMDVVYNH TYNKLMFENI TGQYYTATDL
WP_055733099      YEQRVREFKT MINEFHKAGI RVIMDVVYNH TYNNEMFENI SMQYYTASDL
M. mangrovi       YENRVSEFKT MVDEFHKAGI RVIMDVVYNH TYSKSVFEPI SSSYYTPTDL
                  ** *  **** *  ******* ********** **    ** *         **
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Region I

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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
WP_077400547     SGTGNSIDAN VPMVSRMIRD SLEYWVEEYN IDGFRFDLIG IFDYDEVEEW
WP_027251440     SGTGNSIDAD LPMVSRMIQD SLAYWVDEYG IDGFRFDLIG IFSYQEVEKW
WP_036748130     SGTGNAIDAD QPMVSRMIQD SLAYWVDEYG IDGFRFDLIG IFSYGEVVKW
WP_012487762     SGTGNSIDAD QAMVSRMIQD SLEYWVEEYG IDGFRFDLIG IFSHAEVEKW
WP_028862995     SGTGNSIDAD VPMVSRMIQD SLEFWVDEYG IDGFRFDLIG IFSYQEVEKW
WP_055733099     SGTGNSINAD VPMVSRMIQD SLEYWVDEYS IDGFRFDLIG IFSYQEVQKW
M. mangrovi      SGTGNSIDAN VPMVGRMIRD SLEYWVDEYN IDGFRFDLIG IFDYDEVADW
                 **********   ** *** * **  *****  ********** **   **  *
                                                  Region II
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
WP_077400547     GTHLNATFPD RNLLIYGEPW NGYASDPREL ERVRLGTIGR IHEARVGVFN
WP_027251440     GRALNQQFPD RNLLLYGEPW NGYASDPKEA QRVRYGTTHH MVQEHVGVFN
WP_036748130     GQALNQQFPD RNLLIYGEPW NGYASDPKEA QRVRYGTTHK IAAEHVGVFN
WP_012487762     GRHLNNKFAD RNLLLYGEPW NGYASDAKEG QRVRYGTTRF MVEEHVGVFN
WP_028862995     GHYMNSTFPD RNLLLYGEPW NGYASDPKND QRVRYGTTHN MVEEHVGVFN
WP_055733099     ASHLNSKFAD RNLLIYGEPW NGYASDPLEG QRVRYGTTHN MVDQHVGVFN
M. mangrovi      ADNLNSKFPD RNLLIYGEPW NGFAADSRQP ERVRLGTIGR IHESHAGVFN
                     *  * * **** ***** ** * *      *** **          ****
                                Region III
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
WP_077400547     PKFREAIKGQ NDNGGCNPGD CYAFNNNPDT -WRIEVGSRG ALRYSNNANT
WP_027251440     GAYREALKGS ND--DTRTGY MFNNLDSADA GWSIYDGMRG SAYDPNDSRN
WP_036748130     GAYREALKGS ND--DTRKGF MFNQLDSTDA GWSIYDGIRG SAYDPNDSRN
WP_012487762     GAFREAIKGN ND--GTATAY MFNHVGPADS GWAIYDGLRG SPYNASDSRN
WP_028862995     GAYREAIKGS ND--GTQTGF MFNNLAAADS GWSIYDGLRG SAYDASDSRN
WP_055733099     GAYREALKGS ND--DTRSAY MFNNVAAADS GWAIYDGFRG SPYDANDGRN
M. mangrovi      PKFREAIKGQ NDNGGCNPGD CFALNNNPDT -WRIEVGSRG GLRYTKDKDT
                    *** **  **                     * *  * **

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
WP_077400547     NIDLWDPMFA MDPEQSINYV SAHDNLSLRD KILQWADLNG VSRDS-----
WP_027251440     --STWFRNFA ADPEQSINYI SAHDNFGLWD KVYLSLSGNV VQNSSHQILS
WP_036748130     --STWFRNFA ADPEQSINYI SAHDNFGLWD KVFLSLSSNV VQNSAHQILS
WP_012487762     --GTWFRNFA ADPEQSINYI SAHDNFGLWD KIYLSLATNV VQNSSHQVLS
WP_028862995     --GEWFRNYA ADPEQSINYI SAHDNFGLWD KVYLSLASDV SQNSSHQVNW
WP_055733099     --STWFRNYA ADPEQSINYI SAHDNFALWD KVYLSASSNV VQNSSHQVMS
M. mangrovi      AIDTWDPMFA MDPEQSINYV SAHDNLTLRD KILQWADLNG ISRDS-----
                     *    *  ********  *****  *** *       *
                           Region IV    ###
                                                @
               ....|....| ....|....| ....|....| ....|....| ....|....|
                          710        720        730        740
750
WP_077400547     -------GYL RRIQMFANGI VLTSQGIPFL HGGVELMRDK QED-------
WP_027251440     LTPPASLDYA KRVVNFGMGM VLTSQGIGFV HAGDEFLRTK TDNEQMSDPD
WP_036748130     LTPPVNLDYA KRVVNFGMGM VLTSQGISFV HAGDEFLRTK TDNEHMTVPS
WP_012487762     LSPPANLGYA RRIANFGMGI VLTSQGIPFV HAGDEFLRTK TNNQQIHVAS
WP_028862995     LTPPADLGYA KRVVNFGMGM VLTSQGIPFI HSGDEFLRTK TNNQEMWSAS
WP_055733099     FTPPSDLSYA KRVVNFGMGL VLTSQGIPFI HAGDEFLRTK TNHQQISSPN
M. mangrovi      -------GYL RRIQMFANGI VLTSQGIPFL HGGVELMRDK QED-------
                         *   *  *  *********  *** *  * *
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
WP_077400547     ---------- HNSYQSPDAI NQYYWNWKID NADVYDYYRD VIALRRAHPA
WP_027251440     AWNFGAHGGT HNTYNAPDSF NSIKWQRRAD NAATYDYFKA LIALRRNHAG
WP_036748130     AWNFGHHAGT HNTYNAPDSF NSIKWHRRAD NAATYKYLKD MITLRRHHAG
WP_012487762     AWNHGAHGGT HNTYDAPDSF NSIKWQNKVS NAATFNYFRD LISLRRNHAG
WP_028862995     AWNYGEHGGT HNTYNAPDSF NAVRWADKVD NAPTFNYFKS LISLRRSHAG
WP_055733099     AWNYGDHGGA HNSYNAPDSF NAIRWSNKIA NVATFDYFKQ LIALRRQHAG
M. mangrovi      ---------- HNSYDSPDAI NQYYWQWKID NADIYAYYKD VIALRRAHPA
                            ** *  **   *   *      **    *     * *** *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
WP_077400547     FRLTTWDAVN QHVTSNRP-R YGVMVNHING AAVGDSWSEI LVIYNSADNY
WP_027251440     LRMTSNQDIA SYLTVSRPAE SGGQLVTGLI TYPQDTH-NL FVVYNSGSNQ
WP_036748130     LRMTSNQDIA KYLMVSRPDA FGGQLVTGHI TYPQDTH-NL FVVYNSGDKQ
WP_012487762     LRMNTNAEIA QYLAVARPAE FGGEVVTGHI TNPRDNH-DL FVVYNSGGNR
WP_028862995     MRMNTNQEIA QYMVVDRPDA FAGQVVSAYI TYPEDSK-KL FIVYNSGNNQ
WP_055733099     LRMNSNQEIA QYLSVSRPEQ YAGQVITGHI TDPSDSH-NL FIVYNSGNNQ
M. mangrovi      FRLTSWDAID QHMTSNRP-R YGIVVHHIDG AAVGDSWSEV IVIYNSADNY
                  *               **                             **

                 ....|....| ....|....| ....|....| ....|....| ....
                        860        870        880        890
WP_077400547     THSLPAGEWK VAMEKSDPAA GNGRVVSGSV VAEGTSVTVL YRD-
WP_027251440     TISLPAGDWT LVADAS--GA RQQTGLSGSV VVEGTAVTVF TQAR
WP_036748130     TISLPAGDWT LAVDAS--GA QNQIGLSGNV LVEGTAVTVF TQAR
WP_012487762     YVSLPPGSWT QIADTN--GA THITGISAVA LVEGTAVTVF RKPR
WP_028862995     NITLPSGNWT KAADAN--GA SNISGLSGSA VVEGTAVTVF TQ--
WP_055733099     SITLPAGSWT KVADAN--GA ANAS-VTGST VVEGTAVTMF TQQK
M. mangrovi      TYSLPAGEWK VALEKSDPAA GNGRSVSGSV VAEGTAVTVL YRH-
                    ** * *           *              *** **
```

Fig 5.2: Multiple sequence analysis of pullulanase gene of *Microbulbifer mangrovi* DD-13^{T}. Signatory conserved region I-IV of pullulanase gene is highlighted in yellow colour. The identical residues are marked with *.

(The amino acid sequence of pullulanase of accession number WP_077400547: *Microbulbifer agarilyticus*; WP_027251440: *Photobacterium halotolerans*; WP_036748130: *Photobacterium galatheae*; WP_012487762: *Cellvibrio japonicas* and WP_028862995: *Psychromonas aquimarina*; WP_055733099: *Agarivorans gilvus* were downloaded from NCBI's nr-protein database).

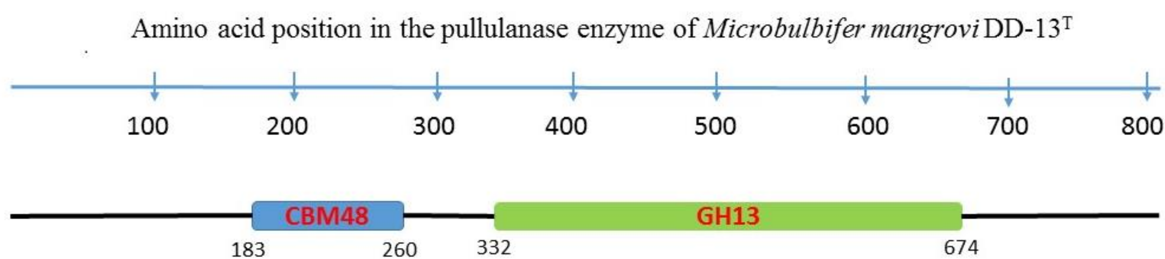
5.3.4 Carbohydrate active enzymes (CAZymes) domain in the pullulanase of *Microbulbifer mangrovi* DD-13^T

Glycoside hydrolase catalytic domain (GH domain) as well as any possible accessory non-catalytic carbohydrate binding modules (CBMs) present in the pullulanase of *Microbulbifer mangrovi* DD-13^T were identified by comparing against CAZy database using dbCan server. Fig 5.3 demonstrate the presence of GH and CBM domains in the pullulanase in *Microbulbifer mangrovi* DD-13^T. The amino acids Phe^{332} to Asn^{674} of DD-13^T pullulanase constitutes the GH13 catalytic domain whereas the amino acid residue from Thr^{183} to Gly^{260} comprised CBM48 domain (Fig 5.3). The non-catalytic carbohydrate binding module, CBM48 would enhance the degradative potential of the cognate catalytic module of pullulanase in DD-13^T. The amino acid residues of pullulanase that constituted the GH13 and CBM48 domains as shown in Fig. 5.1. Based on the detected GH domain, the enzymes were assigned to be in different GH-families of CAZy database (http://www.cazy.org/). The detection of GH13 catalytic domain indicated that the pullulanase of *Microbulbifer mangrovi* DD-13^T belonged to GH13 family of pullulanase.

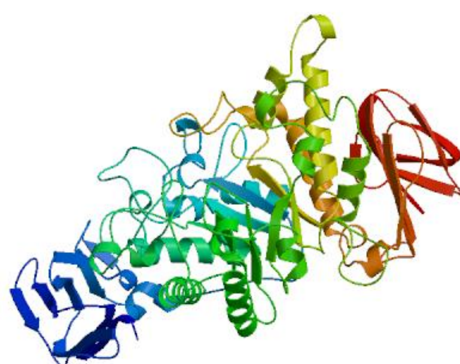
5.3.5 Homology modelling of pullulanase from *Microbulbifer mangrovi* DD-13^T

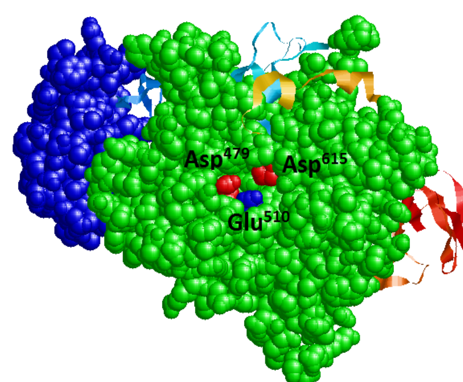
The amino acid sequence of the pullulanase from strain DD-13^T was used to search for the template in SMTL (SWISS model template library). Template search identified many homologous pullulanase and limit dextrinase for pullulanase of strain DD-13^T. The crystal structured pullulanase bearing SMTL id 2yoc.1.A (pullulanase from *Klebsiella oxytoca*), 2fhc.1.A (pullulanase from *Klebsiella pneumoniae*), 2e8z.1.A (Type I pullulanase from *Bacillus subtilis*), 3wdj.1.A (Type I pullulanase from *Anoxybacillus* sp. LM18-11) and 2wan.1.A (pullulanase from *Bacillus acidopullulyticus*) are 27.62%,

27.91%, 33.79%, 34.48% and 36% identical to the pullulanase of strain DD-13^T respectively (Turkenburg et al., 2009; Xu et al., 2014; East et al., 2016; Mikami et al., 2006). Furthermore, limit dextrinase of SMTL id 4j3s.1.A, 4j3w.1.A, 2y5e.1.A are showing 28.55%, 28.55% and 28.71 % identity to the pullulanase from DD-13^T respectively. The pullulanase bearing the SMTL id 2wan.1.A was selected as template to build the model as it depicted maximum identity to the pullulanase from strain DD-13^{T}and had better resolution. The pullulanase of strain DD-13^T was directly submitted to the SwissModel server with 2wan.1.A as the assigned template and a reliable model was obtained (Fig 5.3b). The amino acid residues constituting catalytic triad (Asp^{479}, Glu^{510} and Asp^{615}) in pullulanase of strain DD-13^T were shown in space fill model. Asp residues are shown in red whereas Glu residue is shown in blue (Fig 5.3c). These catalytic residues seems to be closer to each other in 3D model of the strain DD-13^T pullulanase which is feasible for the catalysis. Furthermore, the catalytic GH13 domain and non-catalytic CBM48 domain were visible as two distinct domain supporting the functionally different nature of these domains (Fig 5.3c)



(a)





(b) (c)

Fig 5.3: a) The GH13 and CBM48 domains detected in the pullulanase of *Microbulbifer mangrovi* DD-13^T b) Homology model of pullulanase from *Microbulbifer mangrovi* DD-13^T c) GH13 (green) and CBM48 domains (blue) in the pullulanase of strain DD13^T.

5.3.6 Evolutionary relation of pullulanase of *Microbulbifer mangrovi* DD-13^T with the pullulanase from other bacterial taxa

To infer the evolutionary relation of pullulanase in *Microbulbifer mangrovi* DD-13^T, phylogenetic tree was constructed using MEGA version 7.0. The optimal tree with the sum of branch length = 1.29653542 is shown (Fig 5.4). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)

are shown next to the branches (Fig 5.4). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analysis using neighbour joining method depicts that the pullulanase of DD-13^T clustered with the clade of *Microbulbifer agarilyticus* pullulanase (Fig. 5.4). Similar results were observed for the phylogenetic analysis of pullulanase gene in DD-13^T using maximum likelihood and maximum parsimony methods. This results suggest that the pullulanase in DD-13^T is orthologous to the pullulanase of *Microbulbifer agarilyticus* (WP077400547.1).

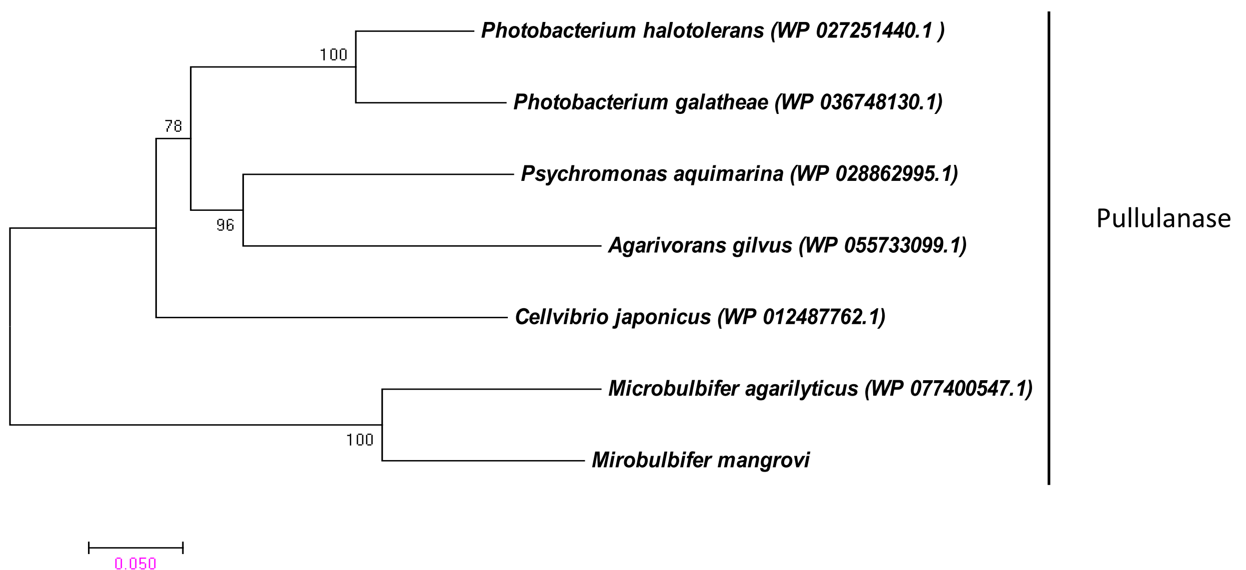


Fig 5.4: Phylogenetic analysis of pullulanase from *Microbulbifer mangrovi* DD-13^T with the homologous pullulanase reported from other bacterial taxa.

5.4 Polysaccharide-degrading genes from *Microbulbifer mangrovi* DD-13^T

As listed in chapter 3, several polysaccharide-degrading genes were identified from the genome of *Microbulbifer mangrovi* DD-13^T indicated the extraordinary potential of strain DD-13^T for the degradation/utilization of various polysaccharides of algal origin/plant origin/animal origin. The genes for polysaccharide-degrading enzymes that were detected in the genome of bacterial strain DD-13^T have been analysed further (Table 5.2)

5.4.1 Agarase genes from *Microbulbifer mangrovi* DD-13^T

Five genes from *Microbulbifer mangrovi* DD-13^T (contig1094:14877-18161; contig1270:133540-135879; contig1270:148758-151022; contig908:82-1140 and contig908:1201-3651) were annotated as agarase genes. The two agarase genes, *aga*(contig908:82-1140) and *aga*(contig908:1201-3651) were located on the forward strand of DD-13^T DNA whereas the other three agarase genes, *aga*(contig1094:14877-18161); *aga*(contig1270:133540-135879) and *aga*(contig1270:148758-151022) are positioned on the reverse strand of the DNA (Table 5.3). The agarase genes of *Microbulbifer mangrovi* DD13^T namely *aga*(contig1094:14877-18161); *aga*(contig1270:133540-135879); *aga*(contig1270:148758-151022); *aga*(contig908:82-1140) and *aga*(contig908:1201-3651) encodes proteins containing 900, 670, 632, 351 and 811 amino acids respectively. The predicted molecular weight of agarase of *Microbulbifer mangrovi* DD-13^T is ranges from 39.57 KDa to 100.27 KDa (Table 5.3). The molecular weight of agarase encoded by *aga*(contig1094:14877-18161) is highest (100.27 KDa) whereas the molecular weight of agarase encoded by *aga*(contig908:82-1140) is lowest (39.57 KDa) amongst the agarases. The predicted pI of agarases of *Microbulbifer mangrovi* DD-13^T ranges from 4.49 to 5.20 (Table 5.3). Furthermore,

Signal P analysis of amino acid sequence of the agarase of *Microbulbifer mangrovi* DD-13^{T} indicate that, the agarase encoded by aga (contig908:1201-3651) has a signal

Table 5.3: General characteristics of polysaccharide-degrading genes identified in the genome of *Microbulbifer mangrovi* DD-13^{T}

Sr. No.	Protein name	Position of genes	ORF detail (Strand), frame	% GC content	No. of amino acids	Predicted Molecular weight (KDa)	Predicted pI
1	Agarase	contig1094: 14877-18161	(-), 1	55.13	900	100.27	4.52
		contig1270: 133540-135879	(-), 1	52.05	670	75.63	5.20
		contig1270: 148758-151022	(-), 1	53.90	632	71.02	4.64
		Contig908: 82-1140	(+), 1	54.39	351	39.57	4.57
		contig908: 1201-3651	(+), 1	54.71	811	89.22	4.49
2	Alginate Lyase	contig1246: 14949-17225	(+), 1	53.36	756	82.48	6.53
		contig1246: 17247-19490	(+), 1	54.94	747	82.69	5.49
3	Carrageenase	contig1270: 97064-101125	(+), 1	55.46	1353	146.08	4.04
4	Chitinase	contig1176: 1453-4569	(-), 1	59.35	1038	110.90	4.15
		contig1212: 35745-36755	(+), 1	54.50	335	37.52	5.22
		contig1270: 61090-62316	(-), 1	57.86	215	23.08	4.25
		contig1270: 62760-65579	(-), 1	59.22	919	98.02	3.91
5	Xylanase	contig884: 105-2843	(-), 1	60.39	873	92.99	4.08
		contig1090: 9803-11326	(-), 1	58.27	295	32.85	4.39
6	Amylase	contig1204: 24298-26052	(+), 1	55.90	543	62.45	5.53
		contig1230: 35822-37561	(-), 1	59.71	579	64.04	4.73
		contig1230:	(+), 1	58.28	647	72.06	4.78

		14935-16878					
		contig1230: 7994-10270	(-), 1	61.18	758	82.24	4.35
7	Pectate Lyase	contig1240: 21784-23556	(-), 1	57.47	449	48.96	4.30
		contig1240: 27163-31128	(-), 1	55.14	1321	137.88	4.41
		contig1240: 14289-15569	(-), 1	58.00	426	45.76	4.58
8	Glycoside hydrolase	contig1024: 3158-4504	(+), 1	59.39	448	49.14	5.47
		contig1116: 17660-19456	(+), 1	53.08	468	53.83	4.91
		contig1186: 19531-20925	(+), 1	58.85	449	50.10	4.24
9	α-Glucurou-ronidase	contig1066: 5042-7222	(-), 1	62.54	726	81.10	5.65
10	Exo-poly-α-D-galacturon oxidase	contig1240:47262-48638	(-), 1	53.96	458	50.53	5.32
11	Endo-1,4-D-glucanase	contig1218: 31916-32779	(-), 1	54.40	287	30.92	4.90
12	β-glucanase	contig1266: 72943-74391	(+), 1	57.69	168	18.67	5.38
13	Glucan 1,4-α-glucosidase	contig1104: 13521-15479	(-), 1	62.84	564	60.33	5.49

peptide of 21 amino acids at N-terminal of the protein. The cleavage site was identified between Ala^{21} and Cys^{22} residues.

The maximum identity depicted by the agarases of *Microbulbifer mangrovi* DD-13^{T} with the agarases from NCBI's nr-protein database is 83%. BlastP analysis indicates that all the five agarases of *Microbulbifer mangrovi* DD-13^{T} depicted identity to the agarases from other *Microbulbifer* sp. and included *Microbulbifer agarilyticus* (WP_077408098.1, WP_010133188.1, AQQ69415.1), *Microbulbifer thermotolerans* (WP_043316972.1), *Microbulbifer* sp. HZ11 (WP_0433169772.1) and *Microbulbifer* sp. Q7 (WP_082859515.1). Furthermore, the agarases of *Microbulbifer mangrovi* DD-13^{T} demonstrated identity to the agarases from other bacterial taxa that includes *Saccharophagus degradans* (WP_011469121.1), *Gilvimarinus agarilyticus* (WP_041522670.1), *Marinomicrobium agarilyticus* (WP_027330171.1) and *Thalassotalea agarivorans* (AGT98631.1). However, the agarases from *Microbulbifer mangrovi* DD-13^{T} depicted no significant identity when they were subjected to pairwise alignment against each other indicating that they are non-homologous to each other.

5.4.2 CAZy classification of agarase from *Microbulbifer mangrovi* DD-13^{T}

The amino acid sequence of agarase of *Microbulbifer mangrovi* DD-13^{T} were analysed for the detection of CAZymes domains. The GH50 domain constituting amino acid residues Lys^{4} – Tyr^{653} was identified in the agarase encoded by aga (contig1270:133540-135879). Similarly, the agarase encoded by aga (contig1270:148758-151022) contain a GH50 domain comprising Thr^{9} – Tyr^{617}. In contrary to these two agarases, two GH86 domains comprising of Thr^{208} – Asp^{240} and Thr^{474} – Lys^{891} were identified in the agarase encoded by aga (contig1094:14877-18161) whereas a single GH86 domain constituting of Asp^{152} – Phe^{811} was detected in the agarase

encoded by the aga contig908:1201-3651). Furthermore, the agarase encoded by aga (contig908:82-1140) contain a GH16 domain comprises of Met^{1} – Tyr^{170} in addition to a CBM6 domain comprise of Asn^{219} – Phe^{324}. Therefore, based on presence of these glycoside hydrolase domain, the agarase encoded by aga (contig1270:133540-135879) and aga (contig1270:148758-151022) are classified as GH50 family agarase whereas the agarase encoded by aga(contig1094:14877-18161) and aga(contig908:1201-3651) belongs to the GH86 family. Additionally, aga (contig908:82-1140) encodes agarase belonging to GH16 family. Furthermore, the agarase belonging to GH16 family is appended with non-catalytic CBM6 (Fig. 5.5). In the phylogenetic tree, the two GH50 agarases grouped together with the bootstrap value of 100% which indicates that both agarases are very closely related. Similarly, the another two agarase belongs to the GH86 family came in the same clade with the bootstrap value of 99 % indicating that the these two GH86 agarase of strain DD-13^{T} are closely related whereas the agarase belongs to the GH16 is relatively distantly related to both GH86 and GH50 agarase of *Microbulbifer mangrovi* DD-13^{T} (Fig 5.5).

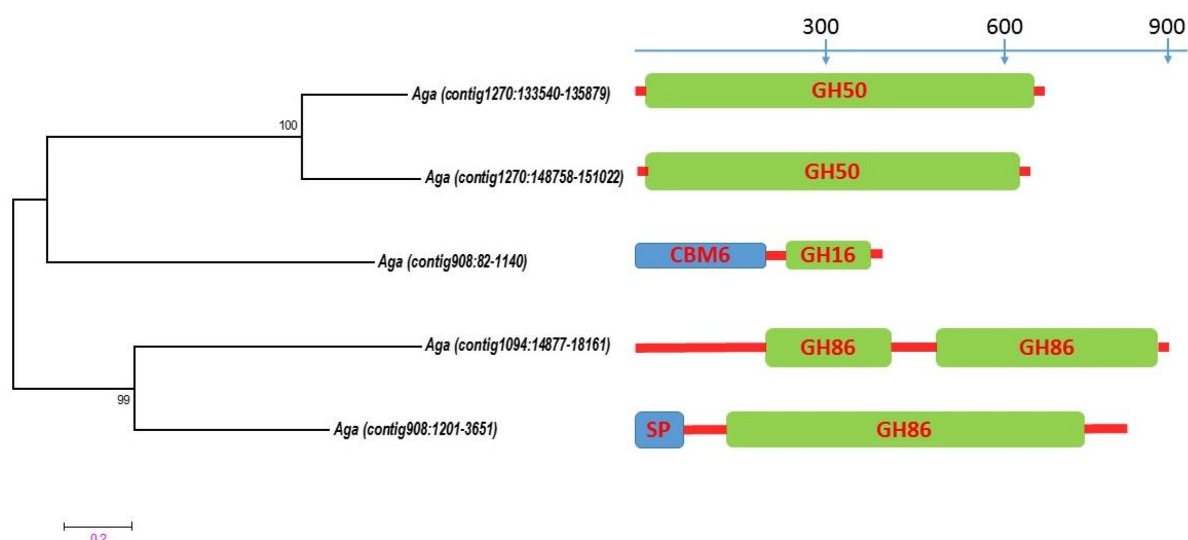


Fig 5.5: Evolutionary relationship between agarases of *Microbulbifer mangrovi* DD-13^{T} belonging to different GH families. GH=Glycoside hydrolase, CBM=Carbohydrate binding modules, SP=Signal peptide. The scale on the top shows position of amino acids in the enzymes.

5.4.3 Alginate lyse genes from *Microbulbifer mangrovi* DD-13^T

Two genes of *Microbulbifer mangrovi* DD-13^T (contig1246:14949-17225; contig1246:17247-19490) were annotated as genes encoding for alginate lyase. Both alginate lyase genes i.e *alg* (contig1246:14949-17225) and *alg* (contig1246:17247-19490) are present on the forward strand of the DNA (Table 5.3). The *alg* (contig1246:14949-17225) and *alg* (contig1246:17247-19490) encodes alginate lyase of 756 and 747 amino acids respectively. The predicted molecular weight of alginate lyase encoded by *alg* (contig1246:14949-17225) and *alg* (contig1246:17247-19490) is 82.48 KDa and 82.69 KDa respectively (Table 5.3). The predicted pI for the alginate lyase encoded by *alg* (contig1246:14949-17225) and *alg* (contig1246:17247-19490) are 6.53 and 5.49 respectively (Table 5.3). Out of the two alginate lyase, one alginate lyase encoded by *alg* (1246:17247-19490) has a signal peptide of 29 amino acids. The cleavage site was identified between Ala^{29} and Met^{30}.

The BLASTp analysis of alginate lyase amino acid sequences encoded by *alg* (contig1246:14949-17225) in *Microbulbifer mangrovi* DD-13^T demonstrated homology to the alginate lyase of many type species of genus *Microbulbifer*. Alginate lyase in DD-13^T is 85 % and 86% identical to the two alginate lyases of *Microbulbifer agarilyticus* WP_081475671.1 and WP_077399235.1 respectively. Additionally, the alginate lyase in DD-13^T shows 69%, 70% and 69% similarity to the alginate lyases of *Microbulbifer thermotolerans* WP_083421109.1, AMX03968.1 and WP_082817332.1 respectively. Alginate lyase from DD-13^T is 83% identical to the alginate lyase from *Microbulbifer donghaiensis* (WP_084536084.1). Furthermore, the alginate lyase in *Microbulbifer mangrovi* DD-13^T also depicts the homology with the alginate lyase from the type species of genus *Colwellia, Shewanella, Pseudoalteromonas* etc. The DD-13^T alginate lyase show 63% similarity to the alginate lyase in *Colwellia aestuarii* (WP_077286922.1),

Colwellia garivorans (WP_077339266.1) and *Colwellia polaris* (WP_085304305.1) whereas 62% similarity to the alginate lyase from *Colwellia sediminilitoris* (WP_085329570.1), *Shewanella waksmanii* (WP_084632515.1) and *Shewanella japonica* (WP_080915131.1). Alginate lyase in DD-13^T is 61% identical to the alginate lyase form *Pseudoalteromonas marina* (WP_021032422.1). Likewise, the DD-13^T alginate lyse is 59% identical to the alginate lyase from *Pseudoalteromonas espejiana* (WP_089348099.1) and *Pseudoalteromonas porphyrae* (WP_054452338.1) whereas 60% identical to the alginate lyase from *Pseudoalteromonas haloplanktis* (WP_002962961.1). Similarly, the alginate lyase encoded by the *alg*(contig1246:17247-19490) in *Microbulbifer mangrovi* DD-13^T shows homology to the alginate lyases of type species of the genus *Microbulbifer*, *Colwellia, Shewanella,* and *Pseudoalteromonas.* It is interesting to note that the both alginate lyase shows similar identity to the alginate lyases of NCBI nr-protein database, however these alginate lyases did not show any significant similarity to each other during pairwise alignment.

5.4.4 CAZy domain in the alginate lyase from *Microbulbifer mangrovi* DD-13^T

Analysis of the amino acid sequence of alginate lyase in DD-13^T against the CAZy database (http://www.cazy.org/) demonstrated that the alginate lyase encoded by the *alg* (contig1246:14949-17225) has two PL6 domains comprising Tyr^{32} – Asn^{401} and Asn^{478} – Thr^{737}. The other alginate lyase of strain DD-13^T encoded by *alg* (contig1246:17247-19490) has two PL17 domains constituting Ala^{191} – Ala^{282} and Gly^{398} – Asn^{533}. Therefore, the alginate lyase encoded by *alg* (contig1246:14949-17225) and *alg* (contig1246:17247-19490) belongs to the PL6 and PL17 families respectively. In CAZy database, the member of PL6 family includes alginate lyase (EC 4.2.2.3), chondroitinase B (EC 4.2.2.19) and MG-specific alginate lyase (EC 4.2.2.-) whereas the PL17 family is exclusively for the alginate lyase/oligo-alginate lyase indicating the

alg(contig1246:14949-17225) encodes alginate lyase whereas *alg*(contig1246:17247-19490) encodes an oligo-alginate lyase. Synergistic action of alginate lyase and oligo alginate lyase could depolymerise the alginate into reducing sugar/s that would be further routed to the various sugar metabolic pathways in strain DD-13^T.

5.4.5 Carrageenase gene from *Microbulbifer mangrovi* DD-13^T

In addition to the genes for agarases and alginate lyase, a single gene for carrageenase is identified in *Microbulbifer mangrovi* DD-13^T genome. The *car* (contig1270:97064-101125) is present on the forward strand of DNA. The carrageenase gene of strain DD-13^T encodes a protein of 1353 amino acids. The predicted molecular weight and pI of carrageenase is 146.08 KDa and 4.04 respectively (Table 5.3).

The carrageenase of *Microbulbifer mangrovi* DD-13^T predominantly shows similarity to the ï-carrageenase from type species of *Cellulophaga* and *Zobellia.* It shows 42% similarity to the ï-carrageenase from *Cellulophaga lytica* (WP_075695576.1), *Cellulophaga qeojensis* (WP_034643654.1), *Cellulophaga fucicola* (WP_072302173.1) and *Zobellia galactanivorans* (WP_013995588.1). Additionally, the carrageenase of DD-13^T depicts 41% similarity to the ï-carrageenase from *Zobellia uliqinosa* (WP_076455364.1) and *Cellulophaga baltica* (WP_0244811070.1). As the carrageenase gene in DD-13^T showing similarity only to the ï-carrageenases from the nr-protein database, indicating the carrageenase of DD-13^T belongs to ï-carrageenase.

5.4.6 CAZymes domain in carrageenase from *Microbulbifer mangrovi* DD-13^T

Furthermore, the alignment of the amino acid sequence carrageenase of *Microbulbifer mangrovi* DD-13^T against the CAZy database depicted the presence of GH82 domain comprises of amino acid residues Val^{916} – Leu^{1099}. Therefore, the carrageenase of *Microbulbifer mangrovi* DD-13^T belongs to GH82 family of CAZy

database. As in CAZy database, the member of the GH82 family belongs to the ï-carrageenase. Thus the carrageenase from strain DD-13^{T} is ï-carrageenase.

5.4.7 Chitinase genes from *Microbulbifer mangrovi* DD-13^{T}

Genome annotation of *Microbulbifer mangrovi* DD-13^{T} reveals the presence of four chitinase encoding genes. The nucleotide sequence contig1176:1453-4569; contig1212:35745-36755; contig1270:61090-62316 and contig1270:62760-65579 were annotated as chitinase. Out of four chitinase genes, three chitinase genes namely chi (contig1176:1453-4569); chi (contig1270:61090-62316) and chi (contig1270:62760-65579) were observed on forward strand whereas chi (contig1212:35745-36755) was located on reverse strand (Table 5.3). The chitinase genes of strain DD-13^{T} i.e., chi (contig1176:1453-4569); chi (contig1212:35745-36755); chi (contig1270:61090-62316) and chi (contig1270:62760-65579) codes for proteins containing 1038, 335, 215 and 919 amino acids respectively (Table 5.3). The predicted molecular weight of chitnase in *Microbulbifer mangrovi* DD-13^{T} ranges from 23.08 KDa to 110.90 KDa. The lowest molecular weight chitinase i.e. 23.08 KDa in *Microbulbifer mangrovi* DD-13^{T} is encoded by chi (contig1270:61090-62316) whereas the highest molecular weight chitinase is encoded by chi (contig1176:1453-4569). The pI of chitinases in *Microbulbifer mangrovi* DD-13^{T} varied from 3.91 to 5.22 (Table 5.3).

BlastP analysis of the four chitinases from *Microbulbifer mangrovi* DD-13^{T} shows identity with the chitinases from different genera. The amino acid sequence of the chitinase encoded by chi (contig1270:62760-65579) depicted 70% - 89% identity with the chitinase of *Microbulbifer* sp. such as *Microbulbifer* sp. Q7 (WP_0669676212.1), *Microbulbifer hydrolyticus* (AAT81212.1), *Microbulbifer agarilyticus* (AQQ69391.1, WP_077401686.1; WP_50901667.1) and *Microbulbifer* sp. ZGT114

(WP_067079543.1). Likewise, the chitinase encoded by chi (contig1212:35745-36755) from *Microbulbifer mangrovi* DD-13^{T} demonstrated 38%-57% identity with the chitinase of type species belonging to genus *Cellvibrio, Parapedobacter, Alqoriphagus and Cyclobacterium* as well as with chitinase from *Cellvibrio japonicas* (WP_012486830.1), *Parapedobacter composti* (SFB82597.1), *Alqoriphagus terriqena* (WP_084454460.1), *Alqoriphagus resistens* (WP_057936748.1), *Alqoriphagus antarcticus* (WP_086542265.1), *Alqoriphagus aquimarinus* (SFB26525.1), *Alqoriphagus halophilus* (SIO15450.1) *Alqoriphagus ratkowski* (WP_086498966.1), *Cyclobacterium Lianum* (WP_084096893.1) and *cyclobacterium halophilum* (WP_92171743.1). Although the chitinase encoded by the chi (contig1174:1453-4569) is 86% identical to the chitinase from *Microbulbifer agarilyticus* (WP_066966359.1) however it depicted only 55% - 57% identity with the chitinase from *Hahella chajuensis* (WP_0113395089.1), *Plesimonas sheiqelloides* (WP_064977297.1, WP_849977889.1, WP_039044655.1), *Reinka blandensis* WP_008042026.1 and *Enterovibrio norvegious* (WP_074925729.1). The chitinase encoded by chi (1270:61090-62316) from strain DD-13^{T} shows 62% - 69% identity with the chitinases from *Stenotrophomonas humi* (WP_67635935.1), *Stenotrophomonas terrae* (WP_057629488.1), *Stenotrophomonas acidominiphila* (WP_054661837.1), *Stenotrophomonas daejeonensis* (WP_083490235.1), *Amantichitinum urcilacus* (WP_083458887.1), *Chromobacterium violaceum* (KUMO3082.1), *Chromobacterium substaqae* (WP_0809624452.1), *Lysobacter qummosus* (WP_0835122943.1) and *Lysobacter antibioticus* (ACN8651.1).

5.4.8 CAZymes domain in chitinases from *Microbulbifer mangrovi* DD-13^{T}

GH18 domains were identified in the chitinases of *Microbulbifer mangrovi* DD13^{T} by aligning the amino acid sequence against the CAZy database. The amino acid residues Arg^{316} – Tyr^{788}; Gly^{2} – Gly^{322}; Met1 – Ala^{114} and Ser^{128} – Asp^{517} constitutes the

GH18 domain in the chitinase encoded by chi (contig1176:1453-4569), chi (contig1212:35745-36755), chi (contig1270:61090-62316) and chi (contig1270:62760-65579) respectively. Therefore all the four chitinases of *Microbulbifer mangrovi* DD-13^T belonged to the GH18 family in CAZy database. Additionally the two chitinases of *Microbulbifer mangrovi* DD-13^T are modular, as the catalytic GH18 domain in the chitinase encoded by chi (contig1176:1453-4569) and chi (contig1270:62760-65579) are appended with two CBM5. The amino acid residues Pro^{31} – Tyr^{66} and Tyr^{993} – Trp^{1032} comprises two CBM5 domain in the chitinase encoded by chi (contig1176:1453-4569) whereas amino acid residues Asp^{827} – Trp^{865} and Pro^{874} – Trp^{910} constitutes the two CBM5 domains in chitinase encoded by chi (1270:62760-65579) (Fig 5.6)

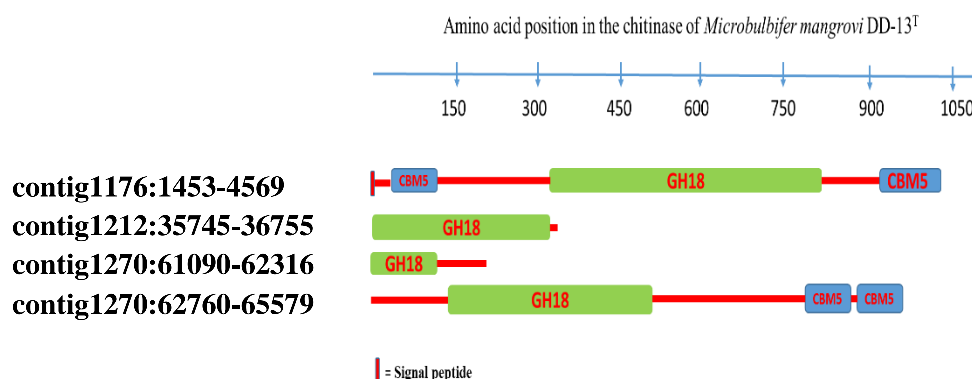


Fig 5.6: GH and CBM domains identified in the chitinases of *Microbulbifer mangrovi* DD-13^T (GH= Glycoside hydrolase; CBM= Carbohydrate binding modules)

5.4.9 Xylanase genes from *Microbulbifer mangrovi* DD-13^T

Two xylanase encoding genes were detected in the genome of *Microbulbifer mangrovi* DD-13^T. Both genes of xylanase viz. xyl (contig1090:9803-11326) and xyl (contig884:105-2843) are located on reverse strand of DNA and encodes proteins of 295 and 873 amino acids respectively (Table 5.3). The molecular weight of xylanase encoded by xyl (contig1090:9803-11326) and xyl (contig884:105-2843) are 32.85 KDa and 92.99

KDa respectively. Signal peptide was not detected in the xylanases encoded by *Microbulbifer mangrovi* DD-13^T.

Blast P analysis indicates that xylanases encoded by xyl (contig884:105-2843) depicts 41 % - 49 % identity predominantly with the Type species of genus *Paenibacillus, Teredinibacter, Cohnella, Gracibacillus* and *clostridium* whereas the xylanase encoded by the xyl (contig1090:9803-11326) demonstrate 60 % - 71 % identity to the 1,4-β-xylanase of several Type species of genus *Teredinibacter.* The low % identity indicates that the xylanase of DD-13^T are novel xylanases.

5.4.10 CAZymes domain in the xylanase from *Microbulbifer mangrovi* DD-13^T

GH10 domain was identified in both the xylanase of *Microbulbifer mangrovi* DD-13^T. The amino acid residues Lys^{134} – Leu^{406} and Leu^{172} – Glu^{502} comprises the GH10 domain in the xylanase encoded by xyl (contig884:105-2843) and xyl (contig1090:9803-11326) respectively. Furthermore, the xylanase of strain DD-13^T is highly modular as the GH10 catalytic domain was appended with the non-catalytic CBM6, and two CBM22 domain in the xylanase encoded by xyl (contig884:105-2843). Additionally the GH10 catalytic domain in xylanase encoded by xyl (contig1090:980-11326) is appended with CBM2 (Fig 5.7). The amino acid residues Ala^{453} – Phe^{555} constitutes the CBM6 domain whereas Asn^{565} – Glu^{693} and Asn^{721} – Gly^{855} comprises the two CBM22 domain identified in the xylanase encoded by xyl (contig1094:9803-11326). Similarly, Ser^{20} – Val^{105} constitutes CBM2 domain in the xylanase encoded by the xyl(contig1090:9803-11326).

Fig 5.7: GHs (Glycoside hydrolases) and CBMs domains identified in the xylanase of *Microbulbifer mangrovi* DD-13^T

5.4.11 Amylase genes from *Microbulbifer mangrovi* DD-13^T

In the genome of strain DD-13^T, four regions i.e. contig1204:24298-26052; contig1230:35822-37561; contig1230:14935-16878 and contig1230:7994-10270 identified to possess amylase encoding genes. Two amylase genes in strain DD-13^T namely amy (contig1204:24298-26052) and amy (contig1230:14935-16878) are located on the forward strand whereas amy (contig1230:7994-10270) and amy (contig1230:35822-37561) were positioned on reverse strand of the DNA (Table 5.3). The amy (contig1204:24298-26052); amy (contig1230:35822-37561); amy (contig1230:14935-16878) and amy (contig1230:7994-10270) genes in strain DD-13^T encodes proteins of 543, 579, 647 and 758 amino acids respectively. The predicted molecular weight of the amylase encoded by amy (contig1204:24298-26052); amy (contig1230:35822-37561); amy (contig1230:14935-16878) and amy (contig1230:7994-10270) are 62.45 KDa, 64.04 KDa, 72.06 KDa and 82.24 KDa respectively. The pI of amylase in DD-13^T varies from 4.35 to 5.53 (Table 5.3).

In all the amylase of *Microbulbifer mangrovi* DD13^T, GH13 domains were identified enabling their classification as GH13 family amylase. The GH13 catalytic

domain appended with CBM10 and CBM2 in the amylase encoded by the amy (contig1090:9803-11326) indicate the highly modular nature of the enzyme.

5.4.12 Pectate lyase/pectinase genes from *Microbulbifer mangrovi* DD-13^T

Three genes, pec (contig1240:21784-23556); pec (contig1240:27163-31128) and pec (contig1240:14289-15569) were annotated as pectate lyase encoding genes that were located on the reverse strand of the DNA (Table 5.3). The pec (contig1240:14289-15569), pec (contig1240:21784-23556) and pec (contig1240:27163-31128) encodes proteins of 426, 449 and 1321 amino acids respectively. The molecular weight of the pectate lyses encoded by the pec (contig1240:14289-15569), pec (contig1240:21784-23556) and pec (contig1240:27163-31128) 45.76 KDa, 48.96 KDa and 137.88 KDa respectively whereas predicted pI are 4.58, 4.30 and 4.41 respectively (Table 5.3).

PL1 domain was detected in the pectinase encoded by pec (contig1240:21784-23556) and pec (contig1240:27163-31128) whereas PL10 domain was identified in the pectinase encoded by pec (contig1240:14289-15569). Furthermore, the catalytic domain in the pectinase/pectate lyase encoded by pec (contig1240:27163-31128) and pec (contig1240:14289-15569) are appended with the CBM35 domain.

5.4.13 Other polysaccharide-degrading genes from *Microbulbifer mangrovi* DD-13^T

In addition to the above mentioned polysaccharide-degrading genes, several other polysaccharide degrading genes were identified in the genome of *Microbulbifer mangrovi* DD-13^T contributing to overall versatility of the bacterium with respect to polysaccharide-degradation. The genes annotated as enzymes involve in polysaccharide-degradation from *Microbulbifer mangrovi* DD-13^T included glycoside hydrolase family 16 domain-containing protein (lichenase, agarase, kappa-carrageenase, endo-β-1,3-glucanase, endo-β-1,3-1,4-glucanase, endo-β-galactosidase, Glycoside hydrolase family

5 (endoglucanase, endomannanase, exoglucanases, exomannanases, β-glucosidase and β-mannosidase), Glycoside hydrolase family 43 (α-L-arabinofuranosidases, endo-α-L-arabinanases, β-D-xylosidases and exo α-1,3-galactanase), Glycoside hydrolase family protein, Arylsulfastase, α-glucuronidase, Exo-poly-α-D-galacturonosidase, Endo-1,4-D-glucanase, β – glucanase, 1,4 β-D-xylanxylohydrolase, Glucan 1,4-α-glucosidase, β-mannosidase, α - L fucosidase, Cellobiohydrolase, Putative glucoamylase I, Xyloglucanase, β –glucosidase, α-glucosidase, β-glycosidase, Arabinan endo-1,5-α-L-arabinosidase and α-N-arabinofuranosidase.

These polysaccharide-degrading enzymes/gene contain various GH/PL catalytic domains and many were appended with at least one CBM (Table 5.4). The Glycoside hydrolase family 5 protein is highly modular as it has two catalytic domain GH5 and GH11. Additionally, two non-catalytic domain CBM5 and CBM10 are also appended to the catalytic domain. Likewise xyloglucanase encoded by contig1036:105-3311 belongs to GH74 and was appended with CBM10 and CBM2.

Table 5.4: Polysaccharide-degrading enzymes of *Microbulbifer mangrovi* DD-13^{T}

Sr. No.	Polysaccharide-degrading enzymes identified in DD-13T genome	Position of polysaccharide-degrading genes in various contings	CAZymes domain detected in polysaccharide-degrading gene/enzymes
9	Glycoside hydrolase	contig1024:3158-4504 contig1116:17660-19456 contig1260:36351-37820	GH28 GH2 -
10	Glycoside hydrolase family 16 domain-containing protein (lichenase, agarase, kappa-carrageenase, endo-β-1,3-glucanase, endo-β-1,3-1,4-glucanase, endo-β-galactosidase	contig1058:6291-8525	GH16|CBM32
11	Glycoside hydrolase family 5 (endoglucanase, endomannanase, exoglucanases, exomannanases, β-glucosidase and β-mannosidase)	contig1186:19531-20925	GH5|CBM10|CBM5|GH11
12	Glycoside hydrolase family 43 (α-L-arabinofuranosidases, endo-α-L-arabinanases, β-D-xylosidases and exo α-1,3-galactanase)	contig1104:10922-12475	GH43
13	Glycoside hydrolase family protein	contig1270:76325-77794	GH16
14	Arylsulfastase	contig1150:13661-15196 contig1150:15278-17521 contig1150:19821-21356 contig1270:73306-74748 contig1270:117398-118831	CBM51 GH43 CBM51 CBM51 CBM51
15	α-glucuronidase	contig1066:5042-7222	GH67
16	Exo-poly-α-D-galacturonosidase	contig1240:47262-48638	GH28
17	Endo-1,4-D-glucanase	contig1218:31916-32779	-
18	β - glucanase	contig1266:72943-74391	CBM6|GH16
19	1,4 β-D-xylanxylohydrolase	contig1236:44604-46211	GH43
20	Glucan 1,4-α-glucosidase	contig1104:2594-19458	GH43
21	β-mannosidase	contig1036:3363-4805	GH5|CBM10
22	α - L fucosidase	contig1158:23994-26477	GH95
23	Cellobiohydrolase	contig1186:21070-22869	GH6|CBM2
24	Putative glucoamylase I	contig1034:8599-9165	-
25	Xyloglucanase	contig1036:105-3311	CBM10|CBM2|GH74
26	β -glucosidase	contig1058:15-1493 contig1102:14996-16333 contig1266:72943-74391	GH3 GH1 CBM6|GH16
27	α-glucosidase	contig1158:12819-14477	GH13
28	β-glycosidase	contig990:4017-5705	GH35
29	Arabinan endo-1,5-α-L-arabinosidase	contig1010:7339-8685	GH43
30	α-N-arabinofuranosidase	contig1110:9285-10262	GH43

5.4.14 CAZymes of *Microbulbifer mangrovi* DD-13^T

The *Microbulbifer mangrovi* DD-13^T encodes a large number of CAZymes including 255 GHs, 17 PLs, 113 CBMs, 145 GTs and 76 Carbohydrate esterase. The GHs and PLs involved in degradation of polysaccharides however the CBMs assist the appended cognate catalytic GH or PL. As seen in Fig. 5.8, 255 genes belonging to the different GH family have been identified in the *Microbulbifer mangrovi* DD-13^T genome. Additionally, in comparison to other multiple polysaccharide-degrading bacteria namely *Microbulbifer elongatus* HZ11, *Microbulbifer thermotolerans* DAU221 and *Saccharophagus degradans* 2-40, *Microbulbifer mangrovi* DD-13^T depicts a wide range of GHs (Fig. 5.8). The most frequent types of GH encoded by strain DD-13^T are GH43 (24), GH109 (19), GH92 (18), GH3 (18), GH23 (16), and GH53 (15) (Fig 5.9a). A total of 30 GHs are appended with at least 1 CBM including 7 GHs which are appended to more than 1 CBM. In addition to GHs, the *Microbulbifer mangrovi* DD-13^T genome encodes a large number of CBMs (113) that is more in number than CBMs reported in other multiple polysaccharide-degrading bacteria *Microbulbifer elongatus* HZ11, *Microbulbifer thermotolerans* DAU221 and *Saccharophagus degradans* 2-40 (Fig 5.8). The genome of strain DD-13^T has frequent numbers of CBM50 (19), CBM2 (17), and CBM32 (13) (Fig 5.9c). Furthermore, 17 PLs identified in *Microbulbifer mangrovi* DD-13^T are distributed to the different PL families namely PL1, PL3, PL6, PL8, PL10 and PL17 family (Fig 5.9b). The PLs identified in *Microbulbifer mangrovi* DD-13^T genome is higher in number than *Microbulbifer elongatus* HZ11 and *Microbulbifer thermotolerans* DAU221 however lower than *Saccharophagus degradans* 2-40 (Fig 5.8)

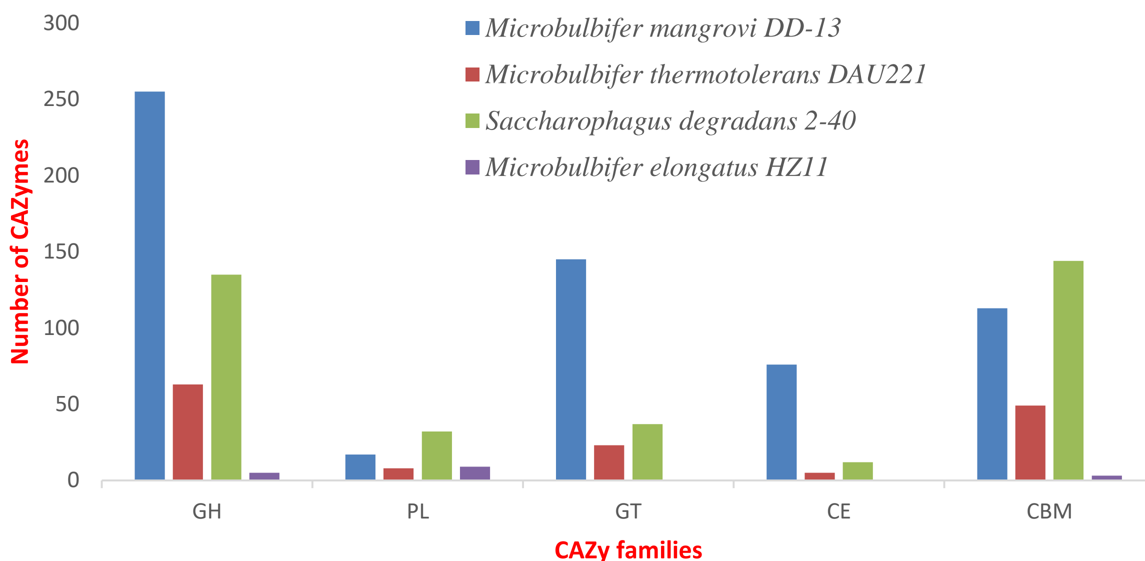
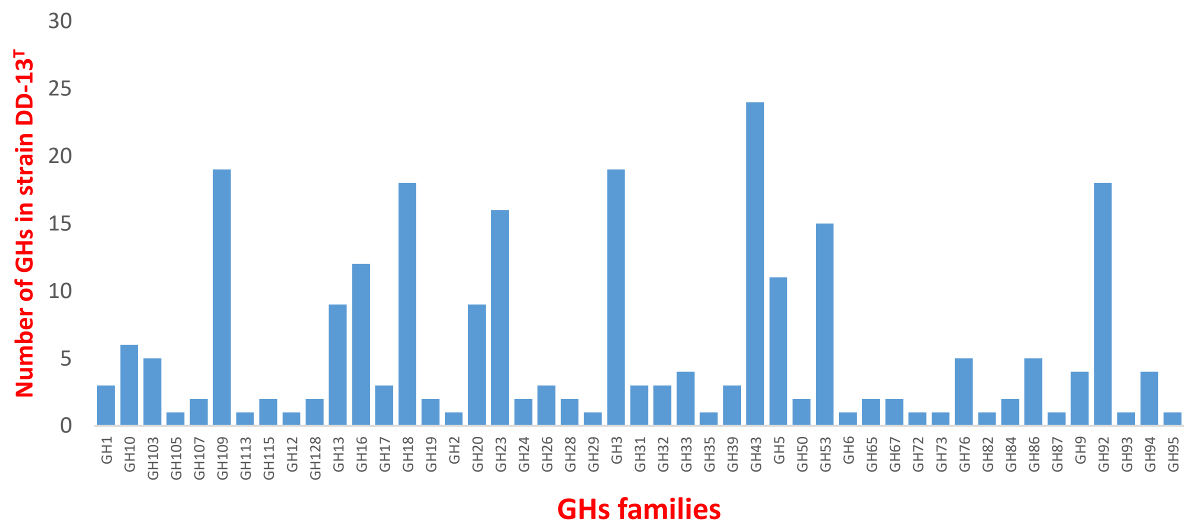
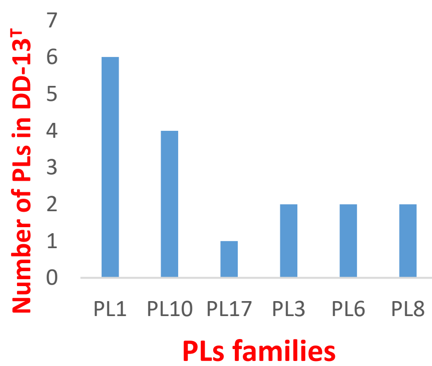


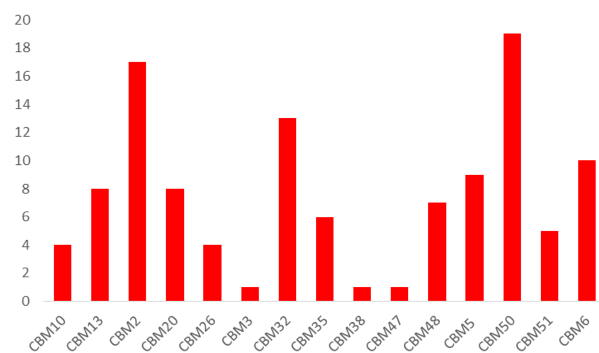
Fig 5.8: Comparison of total number of CAZymes present in the genome of different multiple polysaccharide-degrading bacteria, namely, *Microbulbifer mangrovi* DD-13^{T}, *Microbulbifer thermotolerans* DAU221, *Saccharophagus degradans* 2–40, and *Microbulbifer elongatus* HZ11

(GH glycoside hydrolase, PL polysaccharide lyase, GT glycosyl transferase, CE carbohydrate esterase, CBM carbohydrate-binding module) (the information of CAZymes of *Microbulbifer thermotolerans* DAU221 and *Saccharophagus degradans* 2–40 were taken from the CAZy database)



(a)





(b) (c)

Fig 5.9: Distribution of different CAZymes families detected in the genome of *Microbulbifer mangrovi* DD-13^T. a) Glycoside hydrolase families b) Polysaccharide lyase family c) Carbohydrate binding modules

5.4.15 Application studies of *Microbulbifer mangrovi* DD-13^T with respect to seaweed waste degradation

Microbulbifer mangrovi DD-13^T degraded agarose assisted by extracellular agarase leading to the production of reducing sugars. As observed from Fig. 5.10a, maximum growth was observed at 40 h (OD_{600} 0.155) whereas maximum amounts of agarase activity and reducing sugar were observed at 24 and 40 h, respectively. Alternatively, *Gracilaria* seaweed powder was added to evaluate the potential of strain DD-13^T to degrade seaweed. As seen from Fig. 5.10b, the seaweed powder promoted maximum growth at 240 h (OD_{600} 1.48) whereas a maximum level of reducing sugar and agarase activity was observed at 96 h. Thus, the yield of reducing sugar from 2 g/L agarose was 0.35 g/L.

Strain DD-13^T also degraded alginate with concomitant production of extracellular alginate lyase leading to production of reducing sugar. As seen in Fig. 5.10c, maximum OD was observed at 192 h (OD_{600} 4.2) whereas maximum amounts of alginate lyase activity and reducing sugar were observed at 72 and 144 h, respectively. Also, as seen from Fig. 5.10d, strain DD-13^T also utilized *Sargassum* seaweed powder for its growth. Maximum cell OD was observed at 240 h whereas maximum alginate lyase activity and level of reducing sugar were observed at 72 and 192 h, respectively. The maximum yield of reducing sugar for strain DD-13 using 2 g/L alginate is 0.1 g/L.

The ASW medium supplemented with individual polysaccharides such as carrageenan, xylan, CMC, pectin, pullulan, and starch as a sole carbon source supported the growth of strain DD-13 (Fig 5.11). The respective homologous carbohydrase activity was also detected in the cell-free culture supernatant promoting the degradation of

associated complex polysaccharide to metabolizable reducing sugar for supporting bacterial growth.

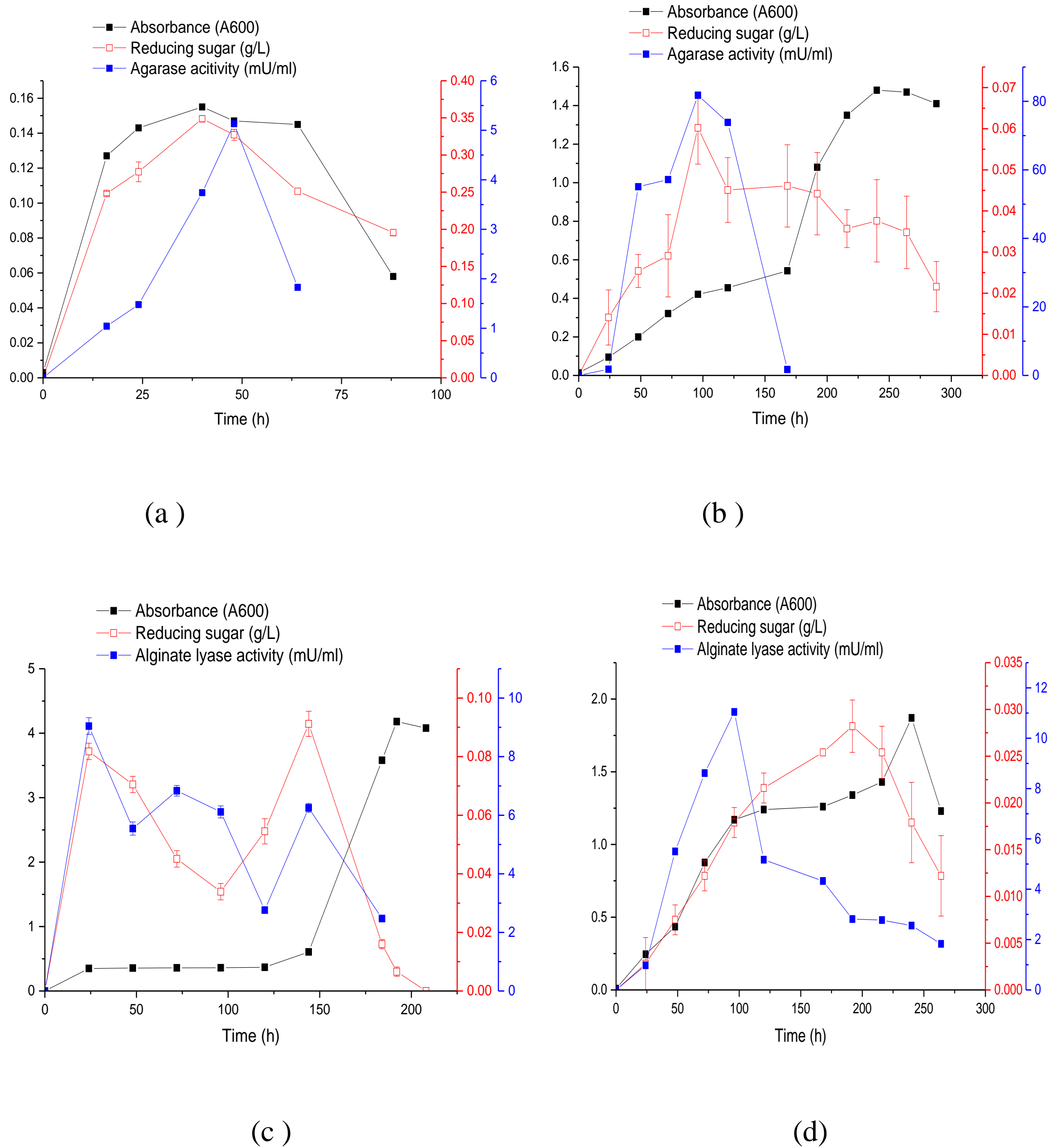


Fig 5.10: Profile of cell growth, reducing sugar, and enzyme activity during growth of *Microbulbifer mangrovi* DD-13^{T} in presence of a) agarose, b) *Gracilaria* seaweed powder, c) alginate, and d) *Sargassum* seaweed powder

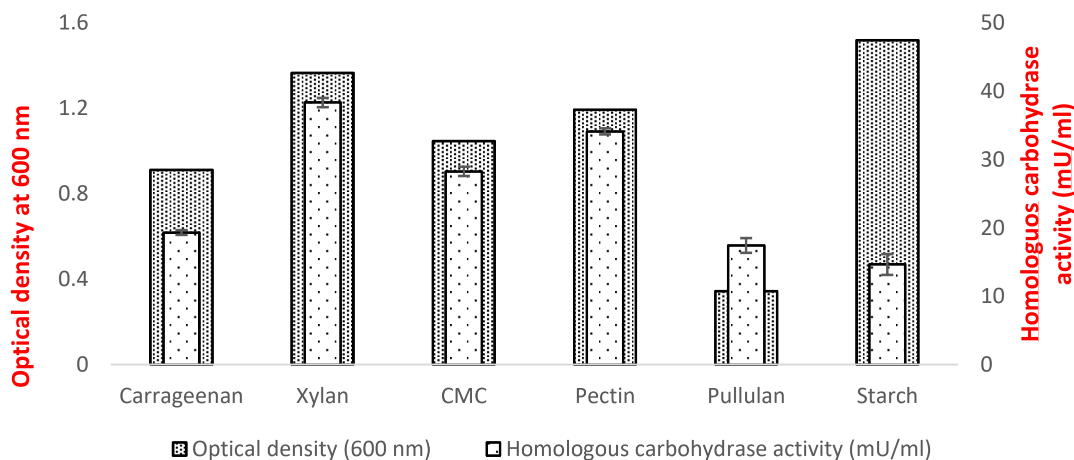


Fig 5.11: Growth of *Microbulbifer mangrovi* DD-13ᵀ and homologous carbohydrase activity at 24 h in the presence of individual polysaccharides as a sole carbon substrate

5.5 Discussion

5.5.1 Pullulanase gene of *Microbulbifer mangrovi* DD-13ᵀ

Pullulanase genes have been cloned from several bacteria and the characterization of nucleotide/amino acid sequence of pullulanase using various bioinformatics tools has been reported. However, present study is a first report on cloning of pullulanase gene and their sequence analysis from *Microbulbifer* sp. The ORF of pullulanase gene in strain DD-13ᵀ is 2415 bp long encoding a protein of 804 amino acids. Previous studies indicates that the size of ORF of pullulanase gene from *Microbulbifer mangrovi* DD-13ᵀ is closer to the size of ORF of type- I pullulanase from *Exigubacterium acetylicum* (2568 bp), *Paenibacillus polymyxa* Nws-pp2 (2532 bp) and *Fervidobacterium pennavorans* Ven5 (2547 bp) (Qiao et al., 2015; Wei et al., 2015; Bertoldo et al., 1999). In contrary, the size of ORF of pullulanase gene identified in *Bacillus megatarium* WW1210 (2814 bp),

Bacillus sp. CICIM263 (2655 bp) is slightly higher to the ORF of pullulanase in DD-13^T whereas the ORF of pullulanase in *Paenibacillus barengoltzii* (2028 bp) is lower in size compare to the ORF of pullulanase in DD-13^T (Yang et al., 2017; Li et al., 2012, Liu et al., 2016). The prediction of molecular weight and pI of protein based on the amino acid sequence frequently enables subsequent analysis of protein by SDS PAGE etc. The ExPasy translation tool has been widely employed to translate the nucleotide sequence to amino acid sequence whereas the ExPasy compute Mw/pI is frequently used by the researchers to predict the molecular weight and pI of the protein based on the amino acid sequence. Using the former, the nucleotide sequence of pullulanase in DD-13^T is translated to a protein of 804 amino acids whereas, molecular weight and pI were predicted using the later. Similarly, the molecular weight and pI of type-I pullulanase of *Paenibacillus polymyxa* Nws-pp2, *Bacillus megaterium* WW1210 and *Bacillus* sp. CICIM 263 were predicted in previous reports (Wei et al., 2015; Yang et al., 2017; Li et al., 2012). The predicted molecular weight of DD13^T pullulanase (90 KDa) is closer to the predicted molecular weight of pullulanase in *Paenibacillus polymyxa* Nws-pp2 (95.6 KDa) and lower than the *Exigubacterium acetylicum* (100 KDa), *Bacillus* sp. CICIM263 (100.8 KDa) and *Bacillus megaterium* WW1210 (106 KDa) (Wei et al., 2015; Qiao et al, 2015; Li et al., 2012; Yang et al., 2017).

The conserved motif YNWGYDP has been reported to present in all type-I pullulanase (Wei et al., 2014; Cai et al., 2014; Doman-Pytka & Bardowski, 2004; Bertoldo et al., 1999; Albertson et al., 1997; D'Elia & Salyers, 1996; Kelly et al., 1994; Kornacker & Pugsley, 1990; Kuriki et al., 1989; Katsuragi et al., 1987) however the variation of one amino acids has been observed in YNWGYDP region of type I pullulanase in *Bacillus stearothermophilus* and *Thermus* sp. (Kuriki et al., 1989; patent WPI 95-100945/14). The conserved region “YNWGYNP” have been reported to present

in the type-I pullulanase of *Bacillus stearothermophilus* and *Thermus* sp. instead of YNWGYDP region (Kuriki et al., 1989; patent WPI 95-100945/14). Our analysis also reveal the presence of YSWGYDP conserved region in the pullulanase of *Microbulbifer mangrovi* DD-13^T. It is interesting to note that, the conserved motif YNWGYDP is restricted to the type-I pullulanase and have been not observed in type II pullulanase. Additionally, the multiple sequence alignment of amino acid sequence have been frequently used to detect the signatory highly conserved regions (region I-IV) in the pullulanase of various bacteria (Wei et al., 2015; Cai et al., 2014; Doman-Pytka et al., 2004; Bertoldo et al., 1999; Albertson et al., 1997). Similarly, in present study, the four conserved region I-IV have been identified in the pullulanase of *Microbulbifer mangrovi* DD-13^T. The conserved region I identified in DD-13^T pullulanase GIRVIMDVVYNHT is exactly similar to the conserved region I identified in the type I pullulanase of *Bacillus thetaiotamicron* whereas closer to the *Fervidobacterium pennavorans* Ven5*, Bacillus acidopullulyticus, Caldicellulosiruptor saccharolyticus, Klebsiella aerogens, Klebsiella pneumoniae* and *Bacillus stearothermophilus* (D'Ellia et al., 1996; Bertoldo et al., 1999; Kelly et al., 1994; Albertson et al., 1997; Katsuragi et al., 1987; Kornacker et al., 1990; Kuriki et al., 1989). Furthermore, the conserved region II (DGFRFDLIG) and conserved region III (YGEPWNG) detected in the DD-13^T pullulanase is very similar to the conserved region II and conserved region III reported in the type I pullulanase of many bacteria (Bertoldo et al., 1999; Albertson et al., 1997; D'Ellia et al., 1996; Kelly et al., 1994; Kornacker et al., 1990; Kuriki et al., 1989; Katsuragi et al., 1987). Previous study indicates that few amino acids are highly conserved in the region I-IV of all type I pullulanase (Wei et al., 2015; Cai et al., 2014; Doman-Pytka et al., 2004; Bertoldo et al., 1999; Albertson et al., 1997; D'Ellia et al., 1996; Kelly et al., 1994; Kornacker et al., 1990; Kuriki et al., 1989; Katsuragi et al., 1987). The "Asp" and "His" residues are

conserved both in the region I and IV of all type I pullulanase whereas "Asp" and "Arg" residues are conserved in region II. Similarly, region III contain the highly conserved "Glu" residue. The conserved region I-IV identified in the pullulanase of DD-13^{T} also contain the identical conserved amino acid residues as reported earlier confirming the classification of the same as type-I pullulanase. The conserved region YNWGYDP along with the conserved region I-IV play a vital role in the catalysis and specifically hydrolyse the α-1,6-glycosidic linkage in pullulan. The conserved acidic amino acid residues of conserved regions in type I pullulanase forms the catalytic triad. The catalytic triads (Asp^{631}, Glu^{660} and Asp^{750}), (Asp^{347}, Glu^{377}, and Asp^{459}) and (Asp^{551}, Glu^{580} and Asp^{671}) are reported to present in the type-I pullulanase from *Bacillus megatarium* WW1210, *Paenibacillus polymyxa* Nws-pp2 and *Exigubacterium acelyticum* (Yang et al., 2017; Wei et al., 2015; Qiao et al., 2015). Similar catalytic triad consisting of Asp^{542}, Glu^{573} and Asp^{679} is also observed in the pullulanase of *Microbulbifer mangrovi* DD-13^{T}. It has been proposed that the hydrolysis of α-1,6-glycosidic linkage follows the retaining mechanism and out of two Asp residues of catalytic triad, one of the Asp residue acts as catalytic nucleophile and other acts as transition state stabilizer while the Glu residue donates the proton (Bertoldo et al. 2004; Domain-Pytka and Bardowski 2004; Kumar 2010). Interestingly, the GH13 catalytic domain identified in the pullulanase of DD-13^{T} ensues the categorization of DD-13^{T} pullulanase as a member of GH13 family in CAZy database. Enzymes belonging to the GH13 families, hydrolyse the glycosidic bond via overall retention of the anomeric configuration further supporting the hypothesis the type-I pullulanase follows retaining mechanism for the hydrolysis of α-1,6- linkage of pullulan (Davies and Henrissat, 1995, Lombard et al., 2014). Furthermore, the catalytic domain GH13 in pullulanase of DD-13^{T} is appended with a non-catalytic carbohydrate binding module 48 (CBM48). CBM increases the degradative potential of cognate catalytic

module by maintaining the proximity with the substrates by increasing the substrate concentration on the surface of the enzymes (Bolam et al. 1998).

Understanding orthology and paralogy are key of protein evolution. As per the definition given by Walter Fitch, orthologs are homologous genes/proteins derived by speciation from a common ancestor whereas homologs derived by duplication is called paralog (Fitch, 1970). Therefore, detection of speciation and duplication process through phylogenetic analysis is ideal to determine the orthology relationship (Gabaldon, 2005) whereas the methods which only rely on sequence similarity levels for the determination of orthology relationship are more prone to error. In present study, the amino acid sequence showing the maximum similarity with the amino acid sequence of DD-13^T pullulanase are consider as homologous sequence to the DD-13 pullulanase and selected to construct a phylogenetic tree to infer the orthology relation of pullulanase in DD-13^T. It was observed from the phylogenetic tree that, pullulanase from DD-13^T has orthologous relation with the pullulanase of *Microbulbifer agarilyticus.*

5.5.2 Other polysaccharide-degrading genes detected in the genome of *Microbulbifer mangrovi* DD-13^T

The polysaccharide-degrading bacteria express a repertoire of hydrolytic enzymes that act in synergy on seaweeds/plant cell wall and other natural polysaccharides to elicit the degradation of often-recalcitrant substrates. The presence of a wide range of polysaccharide-degrading genes include agarase, alginate lyase, carrageenase, amylase, pectinase, xylanase, chitinase etc. reveal the versatility of *Microbulbifer mangrovi* DD-13^T towards the polysaccharide-degradation. The multiple agarase enzymes detected in *Microbulbifer mangrovi* DD-13^T may constitute agar degradation pathway that could generate the reducing/metabolizable sugar from the agar or seaweeds. The

reducing/metabolizable sugar generated as end product may be routed to the various energy conversion pathway operating in the bacterial strain DD-13^T. As reported earlier, agar degradation may follow three different pathway namely α-agarase degradation pathway, β-agarase degradation pathway and β-porphyranase degradation pathway (Chi et al., 2012). In present study, all five agarases annotated as β-agarase suggesting the operation of β-agarase degradation pathway in *Microbulbifer mangrovi* DD-13^T.

β-agarases are classified in four distinct GH families in the CAZy database: GH16, GH50, GH86, and GH118 (Michel et al. 2006). The GH16 is the largest and most heterogeneous family and contain various enzymes including β-agarase. However, the members of GH50, GH86 and GH118 are only β-agarase (Lombard et al., 2014). The detection of GH50, GH86 and GH16 catalytic domain in the agarases of strain DD-13^T confirm their classification in GH16, GH50 and GH86 families and also endorses the annotation of aga (contig1094:14877-18161); aga (contig1270:133540-135879); aga (contig1270:148758-151022); aga (contig908:82-1140) and aga (contig908:1201-3651) as β-agarase. Furthermore, previously many well characterized agarases belonging to the GH16 family produce neoagarotetraose (NA4) or neoagarohexaose (NA6) as the end product (Temuujin et al., 2011; yang et al., 2011; Hehemann et al., 2010a, 2010b; Oh et al., 2010; Long et al., 2010; Lu et al., 2009; Fu et al., 2009; Dong et al., 2007a, 2007b; Zhang and Sun, 2007). Additionally, the GH86 agarase characterized from *Microbulbifer* sp. JAMB-A94 also produces NA6 as the major end product of the reaction (Ohta et al., 2004a). Further, the β-agarase encoded by the aga (contig908:82-1140), aga (contig908:1201-3651) and aga (contig1094:14877-18161) in *Microbulbifer mangrovi* DD-13^T may produce NA4 or NA6 from agar as the end product of the reaction. As per the CAZy database, till date 22 β-agarases belonging to GH50 have been biochemically characterized. The biochemical characterization of GH-50 agarases indicates that they

have either exolytic or both endo- and exolytic activity. Furthermore, biochemical characterization also reveals that the GH50 agarases generates neoagarobiose as a major reaction product from neoagaro-oligosaccharides such as neoagarotetraose and neoagarohexaose or from agarose (Kim et al., 2010; Fu et al., 2008b; Ohta et al., 2005a; Lee et al., 2008; Sugano et al., 1993). Likewise, the NA6 or NA4 produced from agar by the agarase belonging to GH16 and GH86 may be further hydrolysed to the neoagarobiose (NA2) by the GH50 agarase encoded by the aga (contig1270:133540-135879) and aga (contig1270:148758-151022) in strain DD-13^T. For agar to be metabolized by the bacterium, the resulting NA2 must be further hydrolysed into the monomers β-D-galactopyranose (G) and 3,6-anhydro-α-L-galactose (LA) which is reported to accomplished by the enzyme α-neoagarobiose hydrolase (NABH) (Ha et al., 2011; Suzuki et al. 2002). However none of the gene in *Microbulbifer mangrovi* DD-13^T is annotated as NABH, we strongly predict the operation of β-agar degradation pathway in strain DD-13^T based on the presence of five agarases belonging to GH16, GH50 and GH86 families. This prediction is further supported by the growth study of strain DD-13^T on agarose/*Gracilaria* powder as the sole carbon source, measurement of agarase activity during the growth of strain DD-13^T and agarase mediated concomitant production of reducing sugar from the agarose in the growth medium. The growth of *Microbulbifer mangrovi* DD-13^T on agarose or *Gracilaria* sp. powder indicates that strain DD-13^T is able to utilize the agarose. The detection of reducing sugars and the agarase activity in the cell free culture supernatant suggest that strain DD-13^T produces multiple agarases required for the complete hydrolysis of agarose to the metabolizabale sugars that is being utilized by the strain DD-13^T supporting the bacterial growth on agarose/*Gracilaria* powder. The absence of NABH in the strain DD-13^T genome may be justified as the genome sequence is at draft level and few genes are missing in the analysis.

Alginate is the major component of the cell wall of the brown seaweeds. In addition to the five agarases, our analysis results into the identification of two genes annotated as alginate lyase belonging to PL6 and PL17 families in *Microbulbifer mangrovi* DD-13^T. The PL6 family and PL17 family of CAZy database are devoted for the alginate lyase and oligo-alginate lyase/alginate lyase respectively (Lombard et al., 2014). Previously, using the similar approach, nine genes encoding alginate lyase belonging to the PL7, PL6, PL17 and PL18 in *Microbulbifer elongatus* HZ11 were identified (Sun et al., 2014). Seven agarase encoding genes and five genes for alginate lyase were identified in *Microbulbifer* sp. Q7 (Yang et al., 2017). Although, *Microbulbifer elongatus* HZ11 and *Microbulbifer* sp.Q7 have more genes for alginate lyase and agarase, the presence of additional polysaccharide-degrading genes including carrageenase, xylanase, pullulanase, amylase, pectate lyase makes *Microbulbifer mangrovi* DD-13^T more versatile bacterium towards the polysaccharide-degradation in comparison to other *Microbulbifer* sp. The polysaccharide-degrading enzymes comprehend a complex modular architecture comprising discrete modules that are normally joined by relatively unstructured linker sequences (Shoseyov et al., 2006). Interestingly, the catalytic domain (GH/PL domain) of predominant polysaccharide-degrading enzymes including agarase, carrageenase, chitinase, xylanase, pectinase, amylase etc. in *Microbulbifer mangrovi* DD-13^T are appended with CBMs. The catalytic domain increase their degradative capability as the cognate CBMs brings the enzymes into intimate and prolonged association with polysaccharide (Gikes et al., 1988; Teeri et al., 1998; Tomme et al., 1995). Noteworthy that the catalytic domains in two chitinases [chi (contig1176:1453-4569) and chi (contig1270:62760-65579)] in *Microbulbifer mangrovi* DD-13^T is also appended with two CBMs whereas one xylanase [xyl (contig884:61090-62316)] has three CBMs. Furthermore, the one of the gene annotated

as glycoside hydrolase family 5 proteins has four modules; two catalytic domain and two non-catalytic CBMs. This type of high modularity increases the potential these enzymes to acts on recalcitrant polysaccharides as they exhibit very strong affinity towards substrate through avidity effects (Freelove et al. 2001; Boraston et al. 2002).

The presence of multiple polysaccharide-degrading enzymes and various other CAZymes belonging to GHs and PLs families enables the *Microbulbifer mangrovi* DD-13^T, a potential candidate to be used for polysaccharide/seaweeds degradation. The study conducted to ensure the polysaccharide/seaweed degradation demonstrate that *Microbulbifer mangrovi* DD-13^T utilizes agar, alginate, carrageenan, xylan, pullulan, starch, pectin, carboxy methyl cellulose for growth. Additionally, the strain DD-13^T depicts growth on *Gracillaria* and *Sargassum* powder confirming the potential of the bacterium to degrade the green as well as brown seaweeds. Furthermore, the detection of reducing sugars and polysaccharide-degrading enzymes activity in the cell free supernatant indicates that the metabolizable sugars are being produced as result of the action of concomitantly produced polysaccharide-degrading enzymes on the supplemented polysaccharides/seaweeds powder in the growth medium. Further, using dye-based plate qualitative assay, bacterial strain DD-13^T has already been reported to degrade 11 polysaccharides including chitin, laminarin, and β-glucan (Vashist et al. 2013). These findings suggest the strain DD-13^T could be a potential candidate for algal waste degradation and to obtain substrate from algal feedstock for bioethanol production. Previous reports also suggest that, besides their innate role in degradation of CP from litter, the multiple polysaccharide-degrading bacteria have been exploited for degradation of algal wastes and are being acclaimed as a prospective option for generating reducing sugars from complex polysaccharides for biofuel production (Kang and Kim 2015; Kim et al. 2013; Wargacki et al. 2012).

CHAPTER 6

Summary and Conclusion

- Genomic libraries from two multiple polysaccharide-degrading *Microbulbifer* sp. namely *Microbulbifer mangrovi* DD-13^T and *Microbulbifer* strain CMC-5 were prepared using shotgun strategy. The recombinant transformants were screened based on blue white screening strategy.
- The white colonies (recombinant transformants) obtained from genomic library were screened for the polysaccharide-degrading activities using plate based dye/precipitants assay. Approximately 60,000 clones were screened for the degradation of agar, alginate, carrageenan and chitin however none of the clones demonstrated polysaccharides-degrading activity.
- In continuation to our search for polysaccharide-degrading genes, NGS library was prepared from *Microbulbifer mangrovi* DD-13^T and the sequence were reads on Illumina NextSeq500 platform. A total of 68, 62,280 reads with an average length of 151 nucleotides were obtained. These reads were quality filtered.
- The quality filtered reads were assembled into 370 contigs comprising a genome of 4,528,106 bp. The G+C content was 57.15 %. A total of 3749 genes were predicted in the genome of *Microbulbifer mangrovi* DD-13^T using GLIMMER v. 3.02.
- Out of 3749 genes, 3488 (93.0%) were identified as protein-coding genes that demonstrated similarity with proteins from the NCBI nr-database with an e-value of $<1e-5$. The analysis indicates that the 3488 candidate genes of strain DD-13^T have a predicted function and 922 (24.6%) were identified to code for hypothetical proteins whereas 261 (6.96%) predicted proteins did not demonstrate any similarity with any known proteins from the NCBI nr-protein database.

- Based on similarity to COGs, 3348 genes (89.3%) of strain DD-13^T were functionally annotated. In addition to protein-encoding genes, 34 tRNA and 3 rRNA genes were also predicted in strain DD-13^T.
- The genome sequence of *Microbulbifer mangrovi* DD-13^T was further annotated with special emphasis on identification of polysaccharide-degrading genes. Out of 3749 genes of *Microbulbifer mangrovi* DD-13^T, 55 genes were annotated as polysaccharide-degrading genes.
- These 55 polysaccharide-degrading genes were annotated and observed to encode proteins related to diverse polysaccharides-degradation activity indicating the versatility of *Microbulbifer mangrovi*-DD-13^T for polysaccharide-degradation in mangrove ecosystem. In strain DD-13^T genome, five agarase, two alginate lyase and one carrageenase gene were detected. Additionally, four genes for chitinase, two xylanase, one pullulanase, five amylase, three pectate lyase, five arylsulfatase and three β-glucosidase genes were detected. Furthermore, the strain DD-13^T genome encodes singular gene for many other polysaccharide-degrading enzymes including α-glucuronidase, exo-poly-α-d-galacturonosidase, endo-1,4-d-glucanase, β-glucanase, 1,4-β-d-xylanxylohydrolase, glucan-1,4-α-glucosidase, β-mannosidase, α-l-fucosidase, cellobiohydrolase, putative glucoamylase I, xyloglucanase, β-glucosidase, α-glucosidase, β-glycosidase, arabinan endo-1,5-α-l-arabinosidase and α-N-arabinofuranosidase.
- A pullulanase encoding gene was cloned and expressed. The pullulanase encoding ORFs in *Microbulbifer mangrovi* DD-13^T is a stretch of 2415 bp starting from ATG (start codon) and ending with TAA (stop codon) and encoded a protein of 904 amino acids. The pullulanase encoding ORF was cloned in pET-22b and expressed in *E.coli* BL-21 (DE3). The cloning of the gene was confirmed by the

resequencing of the cloned DNA fragment. A proper clone of pullulanase gene of *Microbulbifer mangrovi* DD-13^T was designated as pET22b-Pull and was further used for expression studies.

- The expression of pullulanase gene was induced by 1 mM IPTG in BL21-pET22b-Pull at 18℃. The recombinant pullulanase was 6.54 fold purified from the cell free extract after sonication of BL21-pET22b-Pull using the Ni-NTA agarose column. The purified pullulanase demonstrated a single band of 85 KDa on SDS PAGE.
- The activity of purified recombinant pullulanase was determined by DNSA method. The recombinant pullulanase was recovered with a yield of 62.84 % and specific activity was achieved 30.7 U/mg in comparison to 4.69 U/mg observed in the crude extract of BL21-pET22b-Pull.
- The recombinant pullulanase depicted activity between 30° C to 50° C with maximum pullulanase activity at 45° C. The Pullulanase was observed to lose activity rapidly at 25 to 45°C and was totally inactive at 50°C at 1 h.
- The pullulanase of strain DD-13^T was active in the pH range of 5 -7 whereas highest activity was observed at pH 7. Enzyme lost its activity at pH8. The pullulanase of strain DD-13^T remained stable from pH 5 to pH 7 for 1 h, however pullulanase retain approx. 50 % activity at pH 4 and pH 8 after 1 h.
- After 10 mM EDTA treatment, the purified recombinant pullulanase from strain DD-13^T completely lost its activity in comparison to the control pullulanase (not treated with EDTA). Several agents were evaluated for activity restoration of EDTA treated pullulanase. The Pullulanase activity was almost recovered in the presence of Na ions, whereas 61% activity was recovered in the presence of K

and 42 % activity recovered by Ca and Zn ions. Fe and Cu did not shown any restoration of the pullulanase activity.

- The nucleotide/amino acid sequence of pullulanase was subjected to bioinformatics analysis. The predicted molecular weight of pullulanase is 90 KDa and pI was 4.6. Signal P analysis predicted a putative signal peptide in the pullulanase of *Microbulbifer mangrovi* DD-13^{T} comprised of MIKPTHGGRLRLPAVPCLLLAAFSTSVSA. The cleavage site was observed between Ala^{29} and Glu^{30}.
- The pullulanase of *Microbulbifer mangrovi* DD-13^{T} belonged to GH13 family. The catalytic GH13 domain of DD-13^{T} pullulanase is appended with non-catalytic CBM48 domain that reported to enhance the degradative potential of the cognate catalytic module. The catalytic GH13 domain and non-catalytic CBM48 was observed as two distinct domain in the space fill model of DD-13^{T} pullulanase indicating the functionally different nature of these domains.
- The highly conserved seven amino acids long motif YSWGYDP specific to type I pullulanase is observed in the pullulanase of *Microbulbifer mangrovi* DD-13^{T}. Additionally, four conserved regions (Region I: GIRVIMDVIMDVVYNH; Region II: DGFRFDLIG; Region III: YGEPWNG and Region IV: DPEQSINYVSAHDN) was also identified in DD-13^{T} pullulanase.
- The amino acid residues Tyr^{374}, Asp^{375}, His^{422}, Arg^{477}, Asp^{479}, Leu^{480}, Glu^{510}, Trp^{512}, Val^{543}, Arg^{546}, His^{614}, Asp^{615}, Asn^{616}, His6^{74} and Tyr^{676} constituted the active site whereas the Asp^{479}, Glu^{510}, and Asp^{615} comprised the catalytic site in pullulanase of *Microbulbifer mangrovi* DD-13^{T}. The Asp^{479}, Glu^{510}, and Asp^{615} residues appeared to be closer to each other in space fill model and is essential for the catalysis.

- Phylogenetic analysis indicates that strain DD-13^T pullulanase exhibit orthologous relation with the pullulanase from *Microbulbifer agarilyticus* (WP_077400547.1).
- In addition to the pullulanase, several other polysaccharide-degrading genes were identified from the genome of *Microbulbifer mangrovi* DD-13^T.
- Five agar-degrading genes belonging to GH86, GH50 and GH16 families were identified in the genome of *Microbulbifer mangrovi* DD-13^T. The GH16 agarase is appended with CBM6 module. A putative signal peptides was identified from GH86 agarase encoded by aga (contig908:1201-3651).
- Additionally, the two alginate lyases were identified in the genome of DD-13^T and were classified in the PL6 and PL17 families.
- The carrageenase gene identified in strain DD-13^T belonged to the GH82 family.
- Furthermore, a total of four chitinase genes were identified in strain DD-13^T. These four chitinase genes are classified as GH18 chitinase. The chitinase genes encoded by chi (contig1176:1453-4569) and chi (contig1270:62760-65579) are modular enzymes as the cognate catalytic module is appended with two CBMs domains.
- Two xylanase identified in strain DD-13^T genome is classified under GH10 family. The GH10 catalytic module in the xylanase encoded by xyl (contig884:61090-62316) is appended with two CBM22 modules and one CBM6 domain suggesting the highly modular nature of the enzyme. The xylanase encoded by xyl (contig1270:62760-65579) has one CBM2 domain.
- In strain DD-13^T genome, three genes were annotated as pectate lyase/pectin lyase-like proteins. Out of these three pectate lyases, two belong to the PL1 family while the other comes under the PL10 family and is appended with CBM35.

- Our analysis also reveals that the strain DD-13^T genome encodes five amylases. The four amylases were classified in the GH13 family whereas the fifth is a modular enzyme belonging to the GH10 family that is appended with two carbohydrate-binding modules CBM10 and CBM2.
- Analysis of nucleotide sequence of predicted gene using a Carbohydrate-Active Enzymes (CAZymes) Analysis Toolkit indicates that strain DD-13^T encodes a large set of CAZymes including 255 glycoside hydrolases, 76 carbohydrate esterases, 17 polysaccharide lyases, and 113 carbohydrate-binding modules.
- The most frequent types of GH encoded by strain DD-13^T are GH43 (24), GH109 (19), GH92 (18), GH3 (18), GH23 (16), and GH53 (15). A total of 30 GHs are appended with at least 1 CBM including 7 GHs that are appended to more than 1 CBM. Additionally, the genome of strain DD-13^T has frequent numbers of CBM50 (19), CBM2 (17), and CBM32 (13) whereas 17 PLs identified in strain DD-13^T are distributed to the different PL families such as PL1, PL3, PL6, PL8, PL10 and PL17 family.
- To validate the genomic data, the cell growth of strain DD-13^T was evaluated using pure polysaccharides such as agarose or alginate as carbon source as well as by using red and brown seaweed powder as substrate. The homologous carbohydrase produced by strain DD-13^T during growth degraded the polysaccharide, ensuring the production of metabolizable reducing sugars. Additionally, several other polysaccharides such as carrageenan, xylan, pullulan, pectin, starch, and carboxymethyl cellulose were also corroborated as growth substrate for strain DD-13^T and were associated with concomitant production of homologous carbohydrase.

Conclusion

The pullulanase gene of *Microbulbifer mangrovi* DD-13^{T} is cloned in pET-22b and expressed in *E.coli* BL-21 (DE3). The 85 KDa pullulanase of *Microbulbifer mangrovi* DD-13^{T} is optimally active at 45 ℃ and pH 7.0. Sequence analysis suggest that DD-13^{T} pullulanase is a type-I pullulanase. Further the pullulanase of *Microbulbifer mangrovi* DD-13^{T} belongs to GH13 family. The GH13 domain is appended with CBM48 domains. The amino acid residues Asp^{479}, Glu^{510}, and Asp^{615} constitute the catalytic triad.

Further, the genome sequence of *Microbulbifer mangrovi* DD-13^{T} reveals extraordinary potential for degradation of multiple polysaccharides. The genome harbours a unique array of genes encoding CAZymes for degradation of several complex polysaccharides such as agar, alginate, carrageenan, chitin, cellulose, xylan, pullulan, pectin, etc. The predicted presence of large CAZymes was corroborated by growth studies with various polysaccharides and the capability of the bacterial strain to produce multiple carbohydrases. This is in reasonable agreement with the predicted potential of strain DD-13 to produce multiple CAZymes that empowers the strain to degrade and utilize diverse complex polysaccharides present in the mangrove ecosystem and help in carbon recycling. The capability of the strain to degrade diverse polysaccharides as well as seaweeds indicates its potential to be exploited for the generation of bioactive oligosaccharide derivatives from seaweeds that could be evaluated for applications in medicinal and agricultural fields. Additionally, strain DD-13 has the potential to transform algal and marine polysaccharide wastes to easily metabolizable reducing sugar that in turn can be used as source material for biofuel production

References

Albertson, G. D., McHale, R. H., Gibbs, M. D., & Bergquist, P. L. (1997). Cloning and sequence of a type I pullulanase from an extremely thermophilic anaerobic bacterium, *Caldicellulosiruptor saccharolyticus*. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1354(1), 35-39.

Albrecht, M. T., & Schiller, N. L. (2005). Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Journal of bacteriology*, *187*(11), 3869-3872.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3), 403-410.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*, 25(17), 3389-3402.

Angayarkanni, J., Palaniswamy, M., Murugesan, S., & Swaminathan, K. (2002). Improvement of tea leaves fermentation with *Aspergillus* spp. pectinase. *Journal of bioscience and bioengineering*, 94(4), 299-303.

Annamalai, N., Giji, S., Arumugam, M., & Balasubramanian, T. (2010). Purification and characterization of chitinase from *Micrococcus* sp. AG84 isolated from marine environment. *African Journal of Microbiology Research*, 4(24), 2822-2827.

Annamalai, N., Rajeswari, M. V., Vijayalakshmi, S., & Balasubramanian, T. (2011). Purification and characterization of chitinase from *Alcaligenes faecalis* AU02 by utilizing marine wastes and its antioxidant activity. *Annals of microbiology*, 61(4), 801-807.

Aoki, T., Araki, T., & Kitamikado, M. (1990). Purification and characterization of a novel β-agarase from *Vibrio* sp. AP-2. *The FEBS Journal*, 187(2), 461-465.

Ara, K., Igarashi, K., Saeki, K., & Ito, S. (1995). An alkaline amylopullulanase from alkalophilic *Bacillus* sp. KSM-1378; kinetic evidence for two independent active sites for the α-1, 4 and α-1, 6 hydrolytic reactions. *Bioscience, biotechnology, and biochemistry*, 59(4), 662-666.

Araki, T., Hayakawa, M., Lu, Z., Karita, S., & Morishita, T. (1998). Purification and characterization of agarases from a marine bacterium, *Vibrio* sp. PO-303. *Journal of marine biotechnology*, 6, 260-265.

Araki, T., Higashimoto, Y., & Morishita, T. (1999). Purification and characterization of κ-carrageenase from a marine bacterium, *Vibrio* sp. CA-1004. *Fisheries science*, 65(6), 937-942.

Araki, T., Lu, Z., & Morishita, T. (1998). Optimization of parameters for isolation of protoplasts from *Gracilaria verrucosa* (Rhodophyta). *Journal of marine biotechnology*, 6, 193-197.

Ayadi, D. Z., Ali, M. B., Jemli, S., Mabrouk, S. B., Mezghani, M., Messaoud, E. B., & Bejar, S. (2008). Heterologous expression, secretion and characterization of the *Geobacillus thermoleovorans* US105 type I pullulanase. *Applied microbiology and biotechnology*, 78(3), 473-481.

Barbeyron, T., Gerard, A., Potin, P., Henrissat, B., & Kloareg, B. (1998). The kappa-carrageenase of the marine bacterium *Cytophaga drobachiensis*, Structural and phylogenetic relationships within family-16 glycoside hydrolases. *Molecular biology and evolution*, 15(5), 528-537.

Barbeyron, T., Michel, G., Potin, P., Henrissat, B., & Kloareg, B. (2000). ι-Carrageenases constitute a novel family of glycoside hydrolases, unrelated to that of κ-carrageenases. *Journal of Biological Chemistry*, 275(45), 35499-35505.

Bertoldo, C., Armbrecht, M., Becker, F., Schäfer, T., Antranikian, G., & Liebl, W. (2004). Cloning, sequencing, and characterization of a heat-and alkali-stable type I pullulanase from Anaerobranca gottschalkii. *Applied and environmental microbiology*, 70(6), 3407-3416.

Bertoldo, C., Duffner, F., Jorgensen, P. L., & Antranikian, G. (1999). Pullulanase type I from *Fervidobacterium pennavorans* Ven5: cloning, sequencing, and expression of the gene and biochemical characterization of the recombinant enzyme. *Applied and environmental microbiology*, 65(5), 2084-2091.

Bibel, M., Brettl, C., Gosslar, U., Kriegshäuser, G., & Liebl, W. (1998). Isolation and analysis of genes for amylolytic enzymes of the hyperthermophilic bacterium *Thermotoga maritima*. *FEMS microbiology letters*, 158(1), 9-15.

Bolam, D. N., Ciruela, A., McQueen-Mason, S., Simpson, P., Williamson, M. P., Rixon, J. E., ... & Gilbert, H. J. (1998). *Pseudomonas* cellulose-binding domains mediate their effects by increasing enzyme substrate proximity. *Biochemical journal*, 331(3), 775-781.

Boraston, A. B., Bolam, D. N., Gilbert, H. J., & Davies, G. J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochemical Journal*, 382(3), 769-781.

Boraston, A. B., McLean, B. W., Chen, G., Li, A., Warren, R. A. J., & Kilburn, D. G. (2002). Co-operative binding of triplicate carbohydrate-binding modules from a thermophilic xylanase. *Molecular microbiology*, 43(1), 187-194.

Boyen, C., Bertheau, Y., Barbeyron, T., & Kloareg, B. (1990). Preparation of guluronate lyase from *Pseudomonas alginovora* for protoplast isolation in *Laminaria*. *Enzyme and Microbial Technology*, 12(11), 885-890.

Brown, S. H., & Kelly, R. M. (1993). Characterization of amylolytic enzymes, having both α-1, 4 and α-1, 6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. *Applied and environmental microbiology*, 59(8), 2614-2621.

Brunswick, J. M., Kelly, C. T., & Fogarty, W. M. (1999). The amylopullulanase of *Bacillus* sp. DSM 405. *Applied microbiology and biotechnology*, 51(2), 170-175.

Cai, X., Ma, J., Wei, D. Z., Lin, J. P., & Wei, W. (2014). Functional expression of a novel alkaline-adapted lipase of *Bacillus amyloliquefaciens* from stinky tofu brine and development of immobilized enzyme for biodiesel production. *Antonie Van Leeuwenhoek*, 106(5), 1049-1060.

Chakdar, H., Kumar, M., Pandiyan, K., Singh, A., Nanjappan, K., Kashyap, P. L., & Srivastava, A. K. (2016). Bacterial xylanases: biology to biotechnology. 3 *Biotech*, 6(2), 1-15.

Chao, Y., Wang, S., Wu, S., Wei, J., & Chen, H. (2017). Cloning and Characterization of an Alginate Lyase from Marine *Vibrio.* sp. QD-5. doi: 10.20944/preprints201705.0055.v1

Chavagnat, F., Colette, D. U. E. Z., Guinand, M., Potin, P., Barbeyron, T., Henrissat, B., & Ghuysen, J. M. (1996). Cloning, sequencing and overexpression in *Escherichia coli* of the alginate lyase-encoding aly gene of *Pseudomonas alginovora*: identification of three classes of alginate lyases. *Biochemical Journal*, *319*(2), 575-583.

Chen, J. T., Chen, M. C., Chen, L. L., & Chu, W. S. (2001). Structure and expression of an amylopullulanase gene from *Bacillus stearothermophilus* TS-23. *Biotechnology and applied biochemistry*, 33(3), 189-199.

Chen, X. L., Hou, Y. P., Jin, M., Zeng, R. Y., & Lin, H. T. (2016). Expression and Characterization of a Novel Thermostable and pH-Stable β-Agarase from Deep-Sea Bacterium *Flammeovirga* Sp. OC4. *Journal of agricultural and food chemistry*, 64(38), 7251-7258.

Chi, W. J., Chang, Y. K., & Hong, S. K. (2012). Agar degradation by microorganisms and agar-degrading enzymes. *Applied microbiology and biotechnology*, 94(4), 917-930.

Chi, W. J., Lee, C. R., Dugerjonjuu, S., Park, J. S., Kang, D. K., & Hong, S. K. (2015). Biochemical characterization of a novel iron-dependent GH16 β-agarase, AgaH92, from an agarolytic bacterium *Pseudoalteromonas* sp. H9. *FEMS microbiology letters*, 362(7), fnv035.

Clementi, F. (1997). Alginate production by *Azotobacter vinelandii*. *Critical reviews in biotechnology*, *17*(4), 327-361.

Coleman, R. D., Yang, S. S., & McALISTER, M. P. (1987). Cloning of the debranching-enzyme gene from *Thermoanaerobium brockii* into *Escherichia coli* and *Bacillus subtilis*. *Journal of bacteriology*, 169(9), 4302-4307.

Cotton, L. A., Graham, R. J., & Lee, R. J. (2009). The role of alginate in *P. aeruginosa* PAO1 biofilm structural resistance to gentamicin and ciprofloxacin. *J. Exp. Microbiol. Immunol*, 13, 58-62.

Courtois, J. (2009). Oligosaccharides from land plants and algae: production and applications in therapeutics and biotechnology. *Current Opinion in Microbiology*, 12(3), 261-273.

Cui, F., Dong, S., Shi, X., Zhao, X., & Zhang, X. H. (2014). Overexpression and characterization of a novel thermostable β-Agarase YM01-3, from marine bacterium *Catenovulum agarivorans* YM01T. *Marine drugs*, 12(5), 2731-2747.

Dahiya, N., Tewari, R., & Hoondal, G. S. (2006). Biotechnological aspects of chitinolytic enzymes: a review. *Applied microbiology and biotechnology*, 71(6), 773-782.

Dahiya, N., Tewari, R., Tiwari, R. P., & Hoondal, G. S. (2005). Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World Journal of Microbiology and Biotechnology*, 21(8), 1611-1616.

Davies, G., & Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. Structure, 3(9), 853-859.

Delcher, A. L., Harmon, D., Kasif, S., White, O., & Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. *Nucleic acids research*, 27(23), 4636-4641.

D'Elia, J. N., & Salyers, A. A. (1996). Contribution of a neopullulanase, a pullulanase, and an alpha-glucosidase to growth of Bacteroides thetaiotaomicron on starch. *Journal of bacteriology*, 178(24), 7173-7179.

Din, N., Damude, H. G., Gilkes, N. R., Miller, R. C., Warren, R. A., & Kilburn, D. G. (1994). C1-Cx revisited: intramolecular synergism in a cellulase. *Proceedings of the National Academy of Sciences*, 91(24), 11383-11387.

Doi, H., Chinen, A., Fukuda, H., & Usuda, Y. (2016). *Vibrio algivorus* sp. nov., an alginate-and agarose-assimilating bacterium isolated from the gut flora of a turban shell marine snail. *International Journal of Systematic and Evolutionary Microbiology*, 66(8), 3164-3169.

Domań-Pytka, M., & Bardowski, J. (2004). Pullulan degrading enzymes of bacterial origin. *Critical reviews in microbiology*, 30(2), 107-121.

Dong, G., Vieille, C., & Zeikus, J. G. (1997). Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical

characterization of the recombinant enzyme. *Applied and environmental microbiology*, 63(9), 3577-3584.

Dong, J., Hashikawa, S., Konishi, T., Tamaru, Y., & Araki, T. (2006). Cloning of the novel gene encoding β-agarase C from a marine bacterium, *Vibrio* sp. strain PO-303, and characterization of the gene product. *Applied and environmental microbiology*, 72(9), 6399-6401.

Dong, J., Tamaru, Y., & Araki, T. (2007a). A unique β-agarase, AgaA, from a marine bacterium, *Vibrio* sp. strain PO-303. *Applied microbiology and biotechnology*, 74(6), 1248.

Dong, J., Tamaru, Y., & Araki, T. (2007b). Molecular cloning, expression, and characterization of a β-agarase gene, agaD, from a marine bacterium, *Vibrio* sp. strain PO-303. *Bioscience, biotechnology, and biochemistry*, 71(1), 38-46.

Dong, S., Wei, T. D., Chen, X. L., Li, C. Y., Wang, P., Xie, B. B., ... & Zhang, Y. Z. (2014). Molecular insight into the role of the N-terminal extension in the maturation, substrate recognition, and catalysis of a bacterial alginate lyase from polysaccharide lyase family 18. *Journal of Biological Chemistry*, 289(43), 29558-29569.

Duan, G., Han, F., & Yu, W. (2009). Cloning, sequence analysis, and expression of gene alyPI encoding an alginate lyase from marine bacterium *Pseudoalteromonas* sp. CY24. *Canadian journal of microbiology*, 55(9), 1113-1118.

Duckworth, M., & Turvey, J. R. (1969). An extracellular agarase from a *Cytophaga* species. *Biochemical Journal*, 113(1), 139-142.

East, A., Mechaly, A. E., Huysmans, G. H., Bernarde, C., Tello-Manigne, D., Nadeau, N., ... & Francetic, O. (2016). Structural basis of pullulanase membrane binding and secretion revealed by X-ray crystallography, molecular dynamics and biochemical analysis. *Structure*, 24(1), 92-104.

Ekborg, N. A., Taylor, L. E., Longmire, A. G., Henrissat, B., Weiner, R. M., & Hutcheson, S. W. (2006). Genomic and proteomic analyses of the agarolytic system expressed by *Saccharophagus degradans* 2-40. *Applied and environmental microbiology*, 72(5), 3396-3405.

Elleuche, S., Qoura, F. M., Lorenz, U., Rehn, T., Brueck, T., & Antranikian, G. (2015). Cloning, expression and characterization of the recombinant cold-active type-I pullulanase from *Shewanella arctica*. *Journal of Molecular Catalysis B: Enzymatic*, 116, 70-77.

Erra-Pujada, M., Debeire, P., Duchiron, F., & O'Donohue, M. J. (1999). The type II pullulanase of *Thermococcus hydrothermalis*: molecular characterization of the gene and expression of the catalytic domain. *Journal of bacteriology*, 181(10), 3284-3287.

Fitch, W. M. (1970). Distinguishing homologous from analogous proteins. *Systematic zoology*, 19(2), 99-113.

Flament, D., Barbeyron, T., Jam, M., Potin, P., Czjzek, M., Kloareg, B., & Michel, G. (2007). Alpha-agarases define a new family of glycoside hydrolases, distinct from beta-agarase families. *Applied and environmental microbiology*, 73(14), 4691-4694.

Freelove, A. C., Bolam, D. N., White, P., Hazlewood, G. P., & Gilbert, H. J. (2001). A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of *Piromyces equi*. *Journal of Biological Chemistry*, 276(46), 43010-43017.

Fu, X. T., Lin, H., & Kim, S. M. (2007). Purification and characterization of a Na+/K+ dependent alginate lyase from turban shell gut *Vibrio* sp. YKW-34. *Enzyme and Microbial Technology*, *41*(6), 828-834.

Fu, X. T., Lin, H., & Kim, S. M. (2008). Purification and characterization of a novel β-agarase, AgaA34, from *Agarivorans albus* YKW-34. *Applied microbiology and biotechnology*, 78(2), 265-273.

Fu, X. T., Pan, C. H., Lin, H., & Kim, S. M. (2009). Gene cloning, expression, and characterization of a beta-agarase, agaB34, from *Agarivorans albus* YKW-34. *Journal of microbiology and biotechnology*, 19(3), 257-264.

Fukamizo, T., & Kramer, K. J. (1985). Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect biochemistry*, *15*(2), 141-145.

Furusawa, G., Lau, N. S., Suganthi, A., & Amirul, A. A. A. (2017). Agarolytic bacterium *Persicobacter* sp. CCB-QB2 exhibited a diauxic growth involving galactose utilization pathway. *MicrobiologyOpen,* 6(1).

Gabaldón, T. (2007). Evolution of proteins and proteomes: a phylogenetics approach. *Evolutionary Bioinformatics*, 2005(1), 0-0.

Gacesa, P. (1992). Enzymic degradation of alginates. *International journal of biochemistry*, 24(4), 545-552.

Galperin, M. Y., & Koonin, E. V. (2004). 'Conserved hypothetical'proteins: prioritization of targets for experimental study. *Nucleic acids research*, 32(18), 5452-5463.

Ganghofner, D., Kellermann, J., Staudenbauer, W. L., & Bronnenmeier, K. (1998). Purification and properties of an amylopullulanase, a glucoamylase, and an α-glucosidase in the amylolytic enzyme system of *Thermoanaerobacterium thermosaccharolyticum. Bioscience, biotechnology*, *and biochemistry*, 62(2), 302-308.

Gao, P. J., Chen, G. J., Wang, T. H., Zhang, Y. S., & Liu, J. (2001). Non-hydrolytic Disruption of Crystalline Structure of Cellulose by Cellulose Binding Domain and Linker Sequence of Cellobiohydrolase I from *Penicillium janthinellum*. *Sheng wu hua xue yu sheng wu wu li xue bao Acta biochimica et biophysica Sinica*, 33(1), 13-18.

Gilkes, N. R., Warren, R., Miller, R. C., & Kilburn, D. G. (1988). Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *Journal of Biological Chemistry*, 263(21), 10401-10407.

Giordano, A., Andreotti, G., Tramice, A., & Trincone, A. (2006). Marine glycosyl hydrolases in the hydrolysis and synthesis of oligosaccharides. *Biotechnology journal*, 1(5), 511-530.

Godiska, R., Mead, D., Dhodda, V., Wu, C., Hochstein, R., Karsi, A., ... & Ravin, N. (2009). Linear plasmid vector for cloning of repetitive or unstable sequences in Escherichia coli. *Nucleic acids research*, 38(6), e88-e88.

Gomes, I., Gomes, J., & Steiner, W. (2003). Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization. *Bioresource Technology*, 90(2), 207-214.

González, J. M., Mayer, F., Moran, M. A., Hodson, R. E., & Whitman, W. B. (1997). *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *International Journal of Systematic and Evolutionary Microbiology*, 47(2), 369-376.

Guan, Q., Guo, X., Han, T., Wei, M., Jin, M., Zeng, F., ... & Zhang, S. (2013). Cloning, purification and biochemical characterisation of an organic solvent-, detergent-, and thermo-stable amylopullulanase from *Thermococcus kodakarensis* KOD1. *Process Biochemistry*, 48(5), 878-884.

Guibet, M., Colin, S., Barbeyron, T., Genicot, S., Kloareg, B., Michel, G., & Helbert, W. (2007). Degradation of λ-carrageenan by *Pseudoalteromonas carrageenovora* λ-

carrageenase: a new family of glycoside hydrolases unrelated to κ-and ι-carrageenases. *Biochemical Journal*, 404(1), 105-114.

Guo, S. H., Chen, J. K., & Lee, W. C. (2004). Purification and characterization of extracellular chitinase from *Aeromonas schubertii*. *Enzyme and microbial technology*, *35*(6), 550-556.

Gupta, V., Trivedi, N., Kumar, M., Reddy, C. R. K., & Jha, B. (2013). Purification and characterization of exo-β-agarase from an endophytic marine bacterium and its catalytic potential in bioconversion of red algal cell wall polysaccharides into galactans. *Biomass and bioenergy*, 49, 290-298.

Ha, J. C., Kim, G. T., Kim, S. K., Oh, T. K., Yu, J. H., & Kong, I. S. (1997). β-agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. *Biotechnology and applied biochemistry*, 26(1), 1-6.

Ha, S. C., Lee, S., Lee, J., Kim, H. T., Ko, H. J., Kim, K. H., & Choi, I. G. (2011). Crystal structure of a key enzyme in the agarolytic pathway, α-neoagarobiose hydrolase from *Saccharophagus degradans* 2–40. *Biochemical and biophysical research communications*, 412(2), 238-244.

Hall, J., Black, G. W., Ferreira, L. M., Millward-Sadler, S. J., Ali, B. R., Hazlewood, G. P., & Gilbert, H. J. (1995). The non-catalytic cellulose-binding domain of a novel cellulase from *Pseudomonas fluorescens* subsp. cellulosa is important for the efficient hydrolysis of Avicel. *Biochemical Journal*, 309(3), 749-756.

Han, T., Zeng, F., Li, Z., Liu, L., Wei, M., Guan, Q., ... & Zhang, S. (2013). Biochemical characterization of a recombinant pullulanase from *Thermococcus kodakarensis* KOD1. *Letters in applied microbiology*, 57(4), 336-343.

Han, W., Gu, J., Yan, Q., Li, J., Wu, Z., Gu, Q., & Li, Y. (2012). A polysaccharide-degrading marine bacterium *Flammeovirga* sp. MY04 and its extracellular agarase system. *Journal of Ocean University of China* (English Edition), 11(3), 375-382.

Han, Y., Yang, B., Zhang, F., Miao, X., & Li, Z. (2009). Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with South China Sea sponge *Craniella australiensis*. *Marine Biotechnology*, *11*(1), 132.

Hashimoto, W., Miyake, O., Ochiai, A., & Murata, K. (2005). Molecular identification of *Sphingomonas* sp. A1 alginate lyase (A1-IV′) as a member of novel polysaccharide lyase family 15 and implications in alginate lyase evolution. *Journal of bioscience and bioengineering*, 99(1), 48-54.

Hashimoto, W., Okamoto, M., Hisano, T., Momma, K., & Murata, K. (1998). *Sphingomonas* sp. A1 lyase active on both poly-β-D-mannuronate and heteropolymeric regions in alginate. *Journal of fermentation and bioengineering*, 86(2), 236-238.

Hatada, Y., Igarashi, K., Ozaki, K., Ara, K., Hitomi, J., Kobayashi, T., ... & Ito, S. (1996). Amino acid sequence and molecular structure of an alkaline amylopullulanase from *Bacillus* that hydrolyzes α-1, 4 and α-1, 6 linkages in polysaccharides at different active sites. *Journal of Biological Chemistry*, 271(39), 24075-24083.

Hatada, Y., Ohta, Y., & Horikoshi, K. (2006). Hyperproduction and application of α-agarase to enzymatic enhancement of antioxidant activity of porphyran. *Journal of agricultural and food chemistry*, 54(26), 9895-9900.

Hatada, Y., Saito, K., Hagihara, H., Ozaki, K., & Ito, S. (2001). Nucleotide and deduced amino acid sequences of an alkaline pullulanase from the alkaliphilic bacterium *Bacillus* sp. KSM-1876. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1545*(1), 367-371.

Hehemann, J. H., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., & Michel, G. (2010a). Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature*, 464(7290), 908-912.

Hehemann, J. H., Michel, G., Barbeyron, T., & Czjzek, M. (2010b). Expression, purification and preliminary X-ray diffraction analysis of the catalytic module of a β-agarase from the flavobacterium *Zobellia galactanivorans*. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, 66(4), 413-417.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, 280(2), 309-316.

Hiraga, K., Shou, L., Kitazawa, M., Takahashi, S., Shimada, M., Sato, R., & Oda, K. (1997). Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330. *Bioscience, biotechnology, and biochemistry*, 61(1), 174-176.

Hodgson, D. A., & Chater, K. F. (1981). A Chromosomal Locus Controlling Extracellular Agarase Production by *Streptomyces coelicolor* A 3 (2), and its Inactivation by Chromosomal Integration of Plasmid SCP1. *Microbiology*, 124(2), 339-348.

Hosoya, S., Jang, J. H., Yasumoto-Hirose, M., Matsuda, S., & Kasai, H. (2009). *Psychromonas agarivorans* sp. nov., a novel agarolytic bacterium. *International journal of systematic and evolutionary microbiology*, 59(6), 1262-1266.

Howard, M. B., Ekborg, N. A., Taylor, L. E., Weiner, R. M., & Hutcheson, S. W. (2004). Chitinase B of "*Microbulbifer degradans*" 2-40 contains two catalytic domains with different chitinolytic activities. *Journal of bacteriology*, 186(5), 1297-1303.

Hu, Z., Lin, B. K., Xu, Y., Zhong, M. Q., & Liu, G. M. (2009). Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. HZ105. *Journal of applied microbiology*, 106(1), 181-190.

Huang, L., Zhou, J., Li, X., Peng, Q., Lu, H., & Du, Y. (2013). Characterization of a new alginate lyase from newly isolated *Flavobacterium* sp. S20. *Journal of industrial microbiology & biotechnology*, 40(1), 113-122.

Hutcheson, S. W., Zhang, H., & Suvorov, M. (2011). Carbohydrase systems of *Saccharophagus degradans* degrading marine complex polysaccharides. *Marine drugs*, *9*(4), 645-665.

Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., & Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics*, 11(1), 119.

Hyun, H. H., & Zeikus, J. G. (1985). General biochemical characterization of thermostable pullulanase and glucoamylase from *Clostridium thermohydrosulfuricum*. *Applied and environmental microbiology*, 49(5), 1168-1173.

Imran, M., Poduval, P. B., & Ghadi, S. C. (2017). Bacterial Degradation of Algal Polysaccharides in Marine Ecosystem. In *Marine Pollution and Microbial Remediation* (pp. 189-203). Springer Singapore.

Iwamoto, M., Kurachi, M., Nakashima, T., Kim, D., Yamaguchi, K., Oda, T., ... & Muramatsu, T. (2005). Structure–activity relationship of alginate oligosaccharides in the induction of cytokine production from RAW264. 7 cells. *FEBS letters*, 579(20), 4423-4429.

Jiao, Y. L., Wang, S. J., Lv, M. S., Xu, J. L., Fang, Y. W., & Liu, S. (2011). A GH57 family amylopullulanase from deep-sea *Thermococcus siculi*: expression of the gene

and characterization of the recombinant enzyme. *Current microbiology,* 62(1), 222-228.

Jones, J. D., Grady, K. L., Suslow, T. V., & Bedbrook, J. R. (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *The EMBO journal*, 5(3), 467.

Jonnadula, R., & Ghadi, S. C. (2011). Purification and characterization of β-agarase from seaweed decomposing bacterium *Microbulbifer* sp. strain CMC-5. *Biotechnology and bioprocess engineering*, 16(3), 513-519.

Jonnadula, R., Verma, P., Shouche, Y. S., & Ghadi, S. C. (2009). Characterization of *Microbulbifer* strain CMC-5, a new biochemical variant of *Microbulbifer elongatus* type strain DSM6810T isolated from decomposing seaweeds. *Current microbiology*, *59*(6), 600-607.

Kang, J., Park, K. M., Choi, K. H., Park, C. S., Kim, G. E., Kim, D., & Cha, J. (2011). Molecular cloning and biochemical characterization of a heat-stable type I pullulanase from *Thermotoga neapolitana*. *Enzyme and microbial technology*, 48(3), 260-266.

Kang, N. Y., Choi, Y. L., Cho, Y. S., Kim, B. K., Jeon, B. S., Cha, J. Y., ... & Lee, Y. C. (2003). Cloning, expression and characterization of a β-agarase gene from a marine bacterium, *Pseudomonas* sp. SK38. *Biotechnology letters*, 25(14), 1165-1170.

Kang, S., & Kim, J. K. (2015). Reuse of red seaweed waste by a novel bacterium, *Bacillus* sp. SYR4 isolated from a sandbar. *World Journal of Microbiology and Biotechnology*, 31(1), 209-217.

Katsuragi, N., Takizawa, N., & Murooka, Y. (1987). Entire nucleotide sequence of the pullulanase gene of *Klebsiella aerogenes* W70. *Journal of bacteriology*, 169(5), 2301-2306.

Kawada, A., Hiura, N., Tajima, S., & Takahara, H. (1999). Alginate oligosaccharides stimulate VEGF-mediated growth and migration of human endothelial cells. *Archives of dermatological research,* 291(10), 542-547.

Kelly, A. P., Diderichsen, B., Jorgensen, S., & McConnell, D. J. (1994). Molecular genetic analysis of the pullalanase B gene of *Bacillus acidopullulyticus*. *FEMS microbiology letters*, 115(1), 97-105.

Khambhaty, Y., Mody, K., & Jha, B. (2007). Purification and characterization of κ-carrageenase from a novel γ-proteobacterium, *Pseudomonas elongata* (MTCC 5261) syn. *Microbulbifer elongatus* comb. Nov. *Biotechnology and Bioprocess Engineering*, *12*(6), 668-675.

Khambhaty, Y., Mody, K., & Jha, B. (2008). Purification, characterization and application of a novel extracellular agarase from a marine *Bacillus megaterium*. *Biotechnology and Bioprocess Engineering*, 13(5), 584-591.

Kim, D. E., Lee, E. Y., & Kim, H. S. (2009). Cloning and characterization of alginate lyase from a marine bacterium *Streptomyces* sp. ALG-5. *Marine biotechnology*, 11(1), 10.

Kim, E. J., Fathoni, A., Jeong, G. T., Do Jeong, H., Nam, T. J., Kong, I. S., & Kim, J. K. (2013). *Microbacterium oxydans*, a novel alginate-and laminarin-degrading bacterium for the reutilization of brown-seaweed waste. *Journal of environmental management*, 130, 153-159.

Kim, H. T., Lee, S., Lee, D., Kim, H. S., Bang, W. G., Kim, K. H., & Choi, I. G. (2010). Overexpression and molecular characterization of Aga50D from *Saccharophagus degradans* 2-40: an exo-type β-agarase producing neoagarobiose. *Applied microbiology and biotechnology*, 86(1), 227-234.

Kim, J. H., Sunako, M., Ono, H., Murooka, Y., Fukusaki, E., & Yamashita, M. (2008). Characterization of gene encoding amylopullulanase from plant-originated lactic acid bacterium, *Lactobacillus plantarum* L137. *Journal of bioscience and bioengineering*, 106(5), 449-459.

Kim, J. H., Sunako, M., Ono, H., Murooka, Y., Fukusaki, E., & Yamashita, M. (2009). Characterization of the C-terminal truncated form of amylopullulanase from *Lactobacillus plantarum* L137. *Journal of bioscience and bioengineering*, 107(2), 124-129.

Kim, J., & Hong, S. K. (2012). Isolation and characterization of an agarase-producing bacterial strain, *Alteromonas* sp. GNUM-1, from the West Sea, Korea. *Journal of microbiology and biotechnology*, 22(12), 1621-1628.

Kim, K. J., Yang, Y. J., & Kim, J. G. (2003). Purification and characterization of chitinase from *Streptomyces* sp. M-20. *Journal of biochemistry and molecular biology*, 36(2), 185-189.

Kim, T. J., Park, W. S., Choi, S. K., Yoon, Y. J., Park, K. H., & Kim, J. W. (2000). Molecular cloning and characterization of a thermostable pullulanase from a *Thermus* Strain IM6501. *Food Science and Biotechnology*, 9(3), 188-194.

Kitamikado, M., Tseng, C. H., Yamaguchi, K. U. N. I. K. O., & Nakamura, T. A. K. A. S. H. I. (1992). Two types of bacterial alginate lyases. *Applied and environmental microbiology*, 58(8), 2474-2478.

Kitamikado, M., Yamaguchi, K., Tseng, C. H., & Okabe, B. I. (1990). Method designed to detect alginate-degrading bacteria. *Applied and environmental microbiology*, 56(9), 2939-2940.

Kłosowski, G., Mikulski, D., Czupryński, B., & Kotarska, K. (2010). Characterisation of fermentation of high-gravity maize mashes with the application of pullulanase,

proteolytic enzymes and enzymes degrading non-starch polysaccharides. *Journal of bioscience and bioengineering*, 109(5), 466-471.

Kobayashi, T., Uchimura, K., Miyazaki, M., Nogi, Y., & Horikoshi, K. (2009). A new high-alkaline alginate lyase from a deep-sea bacterium *Agarivorans* sp. *Extremophiles*, *13*(1), 121-129.

Koch, R., Zablowski, P., & Antranikian, G. (1987). Highly active and thermostable amylases and pullulanases from various anaerobic thermophiles. *Applied microbiology and biotechnology*, 27(2), 192-198.

Kornacker, M. G., & Pugsley, A. P. (1990). Molecular characterization of pulA and its product, pullulanase, a secreted enzyme of *Klebsielia pneumoniae* UNF5023. *Molecular microbiology*, 4(1), 73-85.

Kornacker, M. G., & Pugsley, A. P. (1990). The normally periplasmic enzyme β-lactamase is specifically and efficiently translocated through the *Escherichia coli* outer membrane when it is fused to the cell-surface enzyme pullulanase. *Molecular microbiology*, 4(7), 1101-1109.

Kraiwattanapong, J., Ooi, T., & Kinoshita, S. (1997). Cloning and sequence analysis of the gene (alyII) coding for an alginate lyase of *Pseudomonas* sp. OS-ALG-9. *Bioscience, biotechnology, and biochemistry*, 61(11), 1853-1857.

Kraiwattanapong, J., Tsuruga, H., Ooi, T., & Kinoshita, S. (1999). Cloning and sequencing of a *Deleya marina* gene encoding for alginate lyase. *Biotechnology letters*, 21(2), 169-174.

Kriegshäuser, G., & Liebl, W. (2000). Pullulanase from the hyperthermophilic bacterium *Thermotoga maritima*: purification by β-cyclodextrin affinity chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, 737(1), 245-251.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, 33(7), 1870-1874.

Kumar, V. (2010). Analysis of the key active subsites of glycoside hydrolase 13 family members. *Carbohydrate research*, 345(7), 893-898.

Kurachi, M., Nakashima, T., Miyajima, C., Iwamoto, Y., Muramatsu, T., Yamaguchi, K., & Oda, T. (2005). Comparison of the activities of various alginates to induce TNF-α secretion in RAW264. 7 cells. *Journal of infection and chemotherapy*, 11(4), 199-203.

Kurata, A., Nishimura, M., Kishimoto, N., & Kobayashi, T. (2014). Draft genome sequence of a deep-sea bacterium, *Bacillus niacini* strain JAM F8, involved in the degradation of glycosaminoglycans. *Genome announcements*, *2*(5), e00983-14.

Kuriki, T., Park, J. H., & Imanaka, T. (1990). Characteristics of thermostable pullulanase from *Bacillus stearothermophilus* and the nucleotide sequence of the gene. *Journal of fermentation and bioengineering*, 69(4), 204-210.

Kurita, K. (2001). Controlled functionalization of the polysaccharide chitin. *Progress in Polymer science*, *26*(9), 1921-1971.

Kwak, M. J., Song, J. Y., Kim, B. K., Chi, W. J., Kwon, S. K., Choi, S., ... & Kim, J. F. (2012). Genome sequence of the agar-degrading marine bacterium *Alteromonadaceae* sp. strain G7. *Journal of bacteriology*, *194*(24), 6961-6962.

Kwak, Y. S., Akiba, T., & Kudo, T. (1998). Purification and characterization of α-amylase from hyperthermophilic archaeon *Thermococcus profundus*, which hydrolyzes both α-1, 4 and α-1, 6 glucosidic linkages. *Journal of fermentation and bioengineering*, 86(4), 363-367.

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H. H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic acids research*, 35(9), 3100-3108.

Lavín, P., Atala, C., Gallardo-Cerda, J., Gonzalez-Aravena, M., De La Iglesia, R., Oses, R., ... & Laughinghouse IV, H. D. (2016). Isolation and characterization of an Antarctic *Flavobacterium* strain with agarase and alginate lyase activities. *Polish Polar Research*, 37(3), 403-419.

Leach, D., & Lindsey, J. (1986). In vivo loss of supercoiled DNA carrying a palindromic sequence. *Molecular and General Genetics MGG*, 204(2), 322-327.

Leathers, T.D. (2005). Polysaccharides and Polyamides in the Food Industry, Properties, Production, and Patents. Wiley-Vch. Weinheim.

Lee, D. G., Jang, M. K., Lee, O. H., Kim, N. Y., Ju, S. A., & Lee, S. H. (2008). Over-production of a glycoside hydrolase family 50 β-agarase from *Agarivorans* sp. JA-1 in *Bacillus subtilis* and the whitening effect of its product. *Biotechnology letters*, 30(5), 911-918.

Lee, D. G., Park, G. T., Kim, N. Y., Lee, E. J., Jang, M. K., Shin, Y. G., ... & Kim, S. J. (2006). Cloning, expression, and characterization of a glycoside hydrolase family 50 β-agarase from a marine *Agarivorans* isolate. *Biotechnology letters*, 28(23), 1925-1932.

Lee, M. J., Lee, Y. C., & Kim, C. H. (1997). Intracellular and Extracellular Forms of Alkaline Pullulanase from an *AlkaliphilicBacillus* sp. S-1. *Archives of biochemistry and biophysics*, *337*(2), 308-316.

Lee, S. P., Morikawa, M., Takagi, M., & Imanaka, T. (1994). Cloning of the aapT gene and characterization of its product, alpha-amylase-pullulanase (AapT), from

thermophilic and alkaliphilic *Bacillus* sp. strain XAL601. *Applied and environmental microbiology*, 60(10), 3764-3773.

Lee, Y. S., & Choi, Y. L. (2016). Complete genome sequence of cold-adapted enzyme producing *Microbulbifer thermotolerans* DAU221. *Journal of biotechnology*, *229*, 31-32.

Lee, Y., Oh, C., De Zoysa, M., Kim, H., Wickramaarachchi, W. D., Whang, I., ... & Lee, J. (2013). Molecular cloning, overexpression, and enzymatic characterization of glycosyl hydrolase family 16 β-agarase from marine bacterium *Saccharophagus* sp. AG21 in *Escherichia coli*. *Journal of microbiology and biotechnology*, 23(7), 913-922.

Leon, O., Quintana, L., Peruzzo, G., & Slebe, J. C. (1992). Purification and properties of an extracellular agarase from *Alteromonas* sp. strain C-1. *Applied and environmental microbiology*, 58(12), 4060-4063.

Li, J. W., Dong, S., Song, J., Li, C. B., Chen, X. L., Xie, B. B., & Zhang, Y. Z. (2011). Purification and characterization of a bifunctional alginate lyase from *Pseudoalteromonas* sp. SM0524. *Marine drugs*, *9*(1), 109-123.

Li, J., Hu, Q., Li, Y., & Xu, Y. (2015). Purification and characterization of cold-adapted beta-agarase from an Antarctic psychrophilic strain. *Brazilian Journal of Microbiology*, 46(3), 683-690.

Li, S., Jia, P., Wang, L., Yu, W., & Han, F. (2013). Purification and characterization of a new thermostable [kappa]-carrageenase from the marine bacterium *Pseudoalteromonas* sp. QY203. *Journal of Ocean University of China. JOUC*, *12*(1), 155.

Li, S., Wang, L., Han, F., Gong, Q., & Yu, W. (2015). Cloning and characterization of the first polysaccharide lyase family 6 oligoalginate lyase from marine *Shewanella* sp. Kz7. *The Journal of Biochemistry*, 159(1), 77-86.

Li, S., Wang, L., Hao, J., Xing, M., Sun, J., & Sun, M. (2016). Purification and Characterization of a New Alginate Lyase from Marine Bacterium *Vibrio* sp. SY08. *Marine drugs*, 15(1), 1.

Li, S., Yang, X., Zhang, L., Yu, W., & Han, F. (2015). Cloning, expression, and characterization of a cold-adapted and surfactant-stable alginate lyase from marine bacterium *Agarivorans* sp. L11. *J Microbiol Biotechnol*, 25(5), 681-686.

Li, Y., Zhang, L., Niu, D., Wang, Z., & Shi, G. (2012). Cloning, expression, characterization, and biocatalytic investigation of a novel bacilli thermostable type I pullulanase from *Bacillus* sp. CICIM 263. *Journal of agricultural and food chemistry*, 60(44), 11164-11172.

Lin, B., Liu, Y., Lu, G., Zhao, M., & Hu, Z. (2017). An agarase of glycoside hydrolase family 16 from marine bacterium *Aquimarina agarilytica* ZC1. *FEMS Microbiology Letters*, 364(4), fnx012.

Lin, F. P., & Leu, K. L. (2002). Cloning, expression, and characterization of thermostable region of amylopullulanase gene from *Thermoanaerobacter ethanolicus* 39E. *Applied biochemistry and biotechnology*, 97(1), 33-44.

Lin, L. L., Tsau, M. R., & Chu, W. S. (1996). Purification and properties of a 140 kDa amylopullulanase from thermophilic and alkaliphilic *Bacillus* sp. strain TS-23. Biotechnology and applied biochemistry, 24(2), 101-107.

Ling, H., Chuan, L., Rosfarizan, M., & Ariff, A. B. (2009). Characterization of pullulanase type II from Bacillus cereus H1. 5. *American journal of Biochemistry and Biotechnology*, 5(4), 170-179.

Liu, G. L., Li, Y., Chi, Z., & Chi, Z. M. (2011). Purification and characterization of κ-carrageenase from the marine bacterium *Pseudoalteromonas porphyrae* for hydrolysis of κ-carrageenan. *Process biochemistry*, *46*(1), 265-271.

Liu, J., Liu, Y., Yan, F., Jiang, Z., Yang, S., & Yan, Q. (2016). Gene cloning, functional expression and characterisation of a novel type I pullulanase from *Paenibacillus barengoltzii* and its application in resistant starch production. *Protein expression and purification*, 121, 22-30.

Liu, Y., Yi, Z., Cai, Y., & Zeng, R. (2015). Draft genome sequence of algal polysaccharides degradation bacterium, *Flammeovirga* sp. OC4. *Marine genomics*, *21*, 21-22.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., & Henrissat, B. (2013). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic acids research*, 42(D1), D490-D495.

Long, M., Yu, Z., & Xu, X. (2010). A novel β-agarase with high pH stability from marine *Agarivorans* sp. LQ48. *Marine biotechnology*, 12(1), 62-69.

Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic acids research*, 25(5), 955-964.

Lu, X., Chu, Y., Wu, Q., Gu, Y., Han, F., & Yu, W. (2009). Cloning, expression and characterization of a new agarase-encoding gene from marine *Pseudoalteromonas* sp. *Biotechnology letters*, 31(10), 1565-1570.

Ma, C., Lu, X., Shi, C., Li, J., Gu, Y., Ma, Y., ... & Yu, W. (2007). Molecular cloning and characterization of a novel β-agarase, AgaB, from marine *Pseudoalteromonas* sp. CY24. *Journal of Biological Chemistry*, 282(6), 3747-3754.

Ma, Y. X., Dong, S. L., Jiang, X. L., Li, J. & Mou, H. J. (2010). Purification and characterization of K-carrageenase from marine bacterium mutant strain

Pseudoalteromonas Sp. AJ5-13 and its degraded products. *Journal of food biochemistry* 34(3): 661-678

Malagon, F., & Aguilera, A. (1998). Genetic stability and DNA rearrangements associated with a 2× 1.1-Kb perfect palindrome in *Escherichia coli*. *Molecular and General Genetics MGG*, 259(6), 639-644.

Malissard, M., Chavagnat, F., Duez, C., Vacheron, M. J., Guinand, M., Michel, G., & Ghuysen, J. M. (1995). Overproduction and properties of the mannuronate alginate lyase AlxMB. *FEMS microbiology letters*, 126(2), 105-111.

Maloy, S. R. (1990). Experimental techniques in bacterial genetics. *Jones & Bartlett Learning*.

Mann, A. J., Hahnke, R. L., Huang, S., Werner, J., Xing, P., Barbeyron, T., ... & Glöckner, F. O. (2013). The genome of the alga-associated marine flavobacterium *Formosa agariphila* KMM 3901T reveals a broad potential for degradation of algal polysaccharides. *Applied and environmental microbiology*, *79*(21), 6813-6822.

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., ... & Lanczycki, C. J. (2014). CDD: NCBI's conserved domain database. *Nucleic acids research,* 43(D1), D222-D226.

Marotta, M., Martino, A., De Rosa, A., Farina, E., Cartenı, M., & De Rosa, M. (2002). Degradation of dental plaque glucans and prevention of glucan formation using commercial enzymes. *Process Biochemistry*, 38(1), 101-108.

Mathupala, S. P., & Zeikus, J. G. (1993). Improved purification and biochemical characterization of extracellular amylopullulanase from *Thermoanaerobacter ethanolicus* 39E. *Applied microbiology and biotechnology*, 39(4), 487-493.

Mathupala, S. P., Lowe, S. E., Podkovyrov, S. M., & Zeikus, J. G. (1993). Sequencing of the amylopullulanase (apu) gene of *Thermoanaerobacter ethanolicus* 39E, and

identification of the active site by site-directed mutagenesis. *Journal of Biological Chemistry*, 268(22), 16332-16344.

Matsubara, Y., Kawada, R., Iwasaki, K. I., Kimura, Y., Oda, T., & Muramatsu, T. (2000). Cloning and sequence analysis of a gene (aly PG) encoding poly (α-L-guluronate) lyase from *Corynebacterium* sp. strain ALY-1. *Journal of bioscience and bioengineering*, *89*(2), 199-202.

Mei, J., Tang, Z., Yi, Y., Wang, H., Wang, Q., & Ying, G. (2014). Purification and characterization of β-agarase from *Paenibacillus* sp. *Food Science and Biotechnology*, 23(5), 1605-1609.

Melasniemi, H., & Paloheimo, M. (1989). Cloning and expression of the *Clostridium thermohydrosulfuricum* α-amylase-pullulanase gene in *Escherichia coli*. *Microbiology*, 135(6), 1755-1762.

Meng, H., Wang, Z., Meng, X., Xie, L., & Huang, B. (2015). Cloning and expression analysis of the chitinase gene Ifu-chit2 from *Isaria fumosorosea*. *Genetics and molecular biology*, 38(3), 381-389.

Messaoud, E. B., Ammar, Y. B., Mellouli, L., & Bejar, S. (2002). Thermostable pullulanase type I from new isolated *Bacillus thermoleovorans* US105: cloning, sequencing and expression of the gene in *E. coli*. *Enzyme and Microbial Technology*, 31(6), 827-832.

Michaud, P., Da Costa, A., Courtois, B., & Courtois, J. (2003). Polysaccharide lyases: recent developments as biotechnological tools. *Critical reviews in biotechnology*, 23(4), 233-266.

Michel, G., Nyval-Collen, P., Barbeyron, T., Czjzek, M., & Helbert, W. (2006). Bioconversion of red seaweed galactans: a focus on bacterial agarases and carrageenases. *Applied microbiology and biotechnology*, 71(1), 23-33.

Mikami, B., Iwamoto, H., Malle, D., Yoon, H. J., Demirkan-Sarikaya, E., Mezaki, Y., & Katsuya, Y. (2006). Crystal structure of pullulanase: evidence for parallel binding of oligosaccharides in the active site. *Journal of molecular biology*, 359(3), 690-707.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 31(3), 426-428.

Miyake, O., Hashimoto, W., & Murata, K. (2003). An exotype alginate lyase in *Sphingomonas* sp. A1: overexpression in *Escherichia coli*, purification, and characterization of alginate lyase IV (A1-IV). *Protein expression and purification*, 29(1), 33-41.

Miyashita, K., Fujii, T., & Sawada, Y. (1991). Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. Microbiology, 137(9), 2065-2072.

Moh, T. H., Lau, N. S., Furusawa, G., & Amirul, A. A. A. (2017). Complete genome sequence of *Microbulbifer* sp. CCB-MM1, a halophile isolated from Matang Mangrove Forest, Malaysia. *Standards in genomic sciences*, 12(1), 36.

Morrice, L. M., McLEAN, M. W., WIlliamson, F. B., & Long, W. F. (1983). β-agarases I and II from *Pseudomonas atlantica*. *The FEBS Journal*, 135(3), 553-558.

Mou, H., Jiang, X., Liu, Z., & Guan, H. (2004). Structural analysis of kapp-carrageenan oligosaccharides released by carrageenase from marine *Cytophaga* MCA-2. *Journal of food biochemistry*, *28*(3), 245-260.

Muramatsu, T., & Sogi, T. (1990). Characterization of alginate lyases from a marine bacterium. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 97(1), 103-108.

Murao, S., Kawada, T., Itoh, H., Oyama, H., & Shin, T. (1991). Purification and characterization of a novel type of chitinase from *Vibrio alginolyticus* TK-22. *Bioscience, biotechnology, and biochemistry*, 56(2), 368-369.

Murielle, J. A. M., Flament, D., Allouch, J., Potin, P., Thion, L., Kloareg, B., ... & Barbeyron, T. (2005). The endo-β-agarases AgaA and AgaB from the marine bacterium *Zobellia galactanivorans*: two paralogue enzymes with different molecular organizations and catalytic behaviours. *Biochemical Journal*, 385(3), 703-713.

Nair, S. U., Singhal, R. S., & Kamat, M. Y. (2006). Enhanced Production of Thermostable Pullulanase Type 1 Using *Bacillus cereus* FDTA 13 and Its Mutant. *Food Technology & Biotechnology*, 44(2).

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of biological chemistry*, 153(2), 375-380.

Nisha, M., & Satyanarayana, T. (2013). Characterization of recombinant amylopullulanase (gt-apu) and truncated amylopullulanase (gt-apuT) of the extreme thermophile *Geobacillus thermoleovorans* NP33 and their action in starch saccharification. *Applied microbiology and biotechnology*, 97(14), 6279-6292.

Noorwez, S. M., Ezhilvannan, M., & Satyanarayana, T. (2006). Production of a high maltose-forming, hyperthermostable and Ca 2+-independent amylopullulanase by an extreme thermophile *Geobacillus thermoleovorans* in submerged fermentation. *Indian Journal of Biotechnology*, 5:337-345

Oh, C., Nikapitiya, C., Lee, Y., Whang, I., Kang, D. H., Heo, S. J., ... & Lee, J. (2010). Molecular cloning, characterization and enzymatic properties of a novel βeta-agarase from a marine isolate *Psudoalteromonas* sp. AG52. *Brazilian Journal of Microbiology*, 41(4), 876-889.

Oh, C., Nikapitiya, C., Lee, Y., Whang, I., Kim, S. J., Kang, D. H., & Lee, J. (2010). Cloning, purification and biochemical characterization of beta agarase from the marine bacterium *Pseudoalteromonas* sp. AG4. *Journal of industrial microbiology & biotechnology*, 37(5), 483-494.

Oh, Y. H., Jung, C., & Lee, J. (2011). Isolation and characterization of a novel agarase-producing *Pseudoalteromonas* sp. bacterium from the guts of spiny turban shells. *J. Microbiol. Biotechnol*, 21(8), 818-821.

Ohishi, K., Yamagishi, M., Ohta, T., Suzuki, M., Izumida, H., Sano, H., ... & Miwa, T. (1996). Purification and properties of two chitinases from *Vibrio alginolyticus* H-8. *Journal of Fermentation and Bioengineering*, 82(6), 598-600.

Ohta, Y. A., Hatada, Y., Nogi, Y., Miyazaki, M., Li, Z., Akita, M., ... & Horikoshi, K. (2004). Enzymatic properties and nucleotide and amino acid sequences of a thermostable β-agarase from a novel species of deep-sea *Microbulbifer*. *Applied microbiology and biotechnology*, 64(4), 505-514.

Ohta, Y., & Hatada, Y. (2006). A novel enzyme, λ-carrageenase, isolated from a deep-sea bacterium. *Journal of biochemistry*, *140*(4), 475-481.

Ohta, Y., Hatada, Y., Ito, S., & Horikoshi, K. (2005). High-level expression of a neoagarobiose-producing β-agarase gene from *Agarivorans* sp. JAMB-A11 in *Bacillus subtilis* and enzymic properties of the recombinant enzyme. *Biotechnology and applied biochemistry*, 41(2), 183-191.

Ohta, Y., Hatada, Y., Miyazaki, M., Nogi, Y., Ito, S., & Horikoshi, K. (2005). Purification and Characterization of a Novel α-Agarase from a *Thalassomonas* sp. *Current microbiology*, 50(4), 212-216.

Ohta, Y., Hatada, Y., Nogi, Y., Li, Z., Ito, S., & Horikoshi, K. (2004). Cloning, expression, and characterization of a glycoside hydrolase family 86 β-agarase from a

deep-sea *Microbulbifer*-like isolate. *Applied microbiology and biotechnology*, 66(3), 266-275.

Ohta, Y., Nogi, Y., Miyazaki, M., Li, Z., Hatada, Y., ItO, S., & Horikoshi, K. (2004). Enzymatic properties and nucleotide and amino acid sequences of a thermostable β-agarase from the novel marine isolate, JAMB-A94. *Bioscience, biotechnology, and biochemistry*, 68(5), 1073-1081.

Okazaki, K., Kawabata, T., Nakano, M., & Hayakawa, S. (1999). Purification and properties of chitinase from *Arthrobacter* sp. NHB-10. *Bioscience, biotechnology, and biochemistry*, 63(9), 1644-1646.

Olsen, H. S. (2000). Enzymes at Work: A Concise Guide to Industrial Enzymes and their Uses. *Novo Nordisk A/S*.

Oranusi, N. A., & Trinci, A. P. (1985). Growth of bacteria on chitin, fungal cell walls and fungal biomass, and the effect of extracellular enzymes produced by these cultures on the antifungal activity of amphotericin B. *Microbios*, 43(172), 17-30.

Park, B. H., Karpinets, T. V., Syed, M. H., Leuze, M. R., & Uberbacher, E. C. (2010). CAZymes Analysis Toolkit (CAT): web service for searching and analyzing carbohydrate-active enzymes in a newly sequenced organism using CAZy database. *Glycobiology*, 20(12), 1574-1584.

Park, D., Jagtap, S., & Nair, S. K. (2014). Structure of a PL17 family alginate lyase demonstrates functional similarities among exotype depolymerases. *Journal of Biological Chemistry*, 289(12), 8645-8655.

Park, H. H., Kam, N., Lee, E. Y., & Kim, H. S. (2012). Cloning and characterization of a novel oligoalginate lyase from a newly isolated bacterium *Sphingomonas* sp. MJ-3. *Marine biotechnology*, 14(2), 189-202.

Park, S.H., Lee, J. & Lee, H.K. (2000). (2000). Purification and characterization of chitinase from a marine bacterium, *Vibrio* sp. 98CJ11027. *The Journal of Microbiology*. 38(4): 224–229

Plant, A. R., Clemens, R. M., Daniel, R. M., & Morgan, H. W. (1987). Purification and preliminary characterization of an extracellular pullulanase from *Thermoanaerobium* Tok6-B1. *Applied microbiology and biotechnology*, 26(5), 427-433.

Pope, A. M., & Davies, D. A. (1979). The influence of carbohydrases on the growth of fungal pathogens in vitro and in vivo. *Postgraduate medical journal*, 55(647), 674-676.

Potin, P., Richard, C., Rochas, C., & Kloareg, B. (1993). Purification and characterization of the α-agarase from *Alteromonas agarlyticus* (Cataldi) comb. nov., strain GJ1B. *The FEBS Journal*, 214(2), 599-607.

Potin, P., Sanseau, A., Gall, Y., Rochas, C., & Kloareg, B. (1991). Purification and characterization of a new k-carrageenase from a marine *Cytophaga*-like bacterium. *The FEBS Journal*, 201(1), 241-247.

Qiao, Y., Peng, Q., Yan, J., Wang, H., Ding, H., & Shi, B. (2015). Gene cloning and enzymatic characterization of alkali-tolerant type I pullulanase from *Exiguobacterium acetylicum. Letters in applied microbiology*, 60(1), 52-59.

Ramesh, M. V., Podkovyrov, S. M., Lowe, S. E., & Zeikus, J. G. (1994). Cloning and sequencing of the *Thermoanaerobacterium saccharolyticum* B6A-RI apu gene and purification and characterization of the amylopullulanase from *Escherichia coli*. *Applied and environmental microbiology*, 60(1), 94-101.

Ramli, A. N., Mahadi, N. M., Rabu, A., Murad, A. M., Bakar, F. D., & Illias, R. M. (2011). Molecular cloning, expression and biochemical characterisation of a cold-

adapted novel recombinant chitinase from *Glaciozyma antarctica* PI12. *Microbial cell factories*, 10(1), 94.

Rendleman, J. A. (1997). Enhancement of cyclodextrin production through use of debranching enzymes. *Biotechnology and applied biochemistry*, 26(1), 51-61.

Revathi, M., Saravanan, R., & Shanmugam, A. (2012). Production and characterization of chitinase from *Vibrio* species, a head waste of shrimp *Metapenaeus dobsonii* (Miers, 1878) and chitin of Sepiella inermis Orbigny, 1848. *Advances in Bioscience and Biotechnology*, *3*(04), 392.

Roh, H., Yun, E. J., Lee, S., Ko, H. J., Kim, S., Kim, B. Y., ... & Choi, I. G. (2012). Genome sequence of *Vibrio* sp. strain EJY3, an agarolytic marine bacterium metabolizing 3, 6-anhydro-L-galactose as a sole carbon source. *Journal of bacteriology*, *194*(10), 2773-2774.

Roy, A., Messaoud, E. B., & Bejar, S. (2003). Isolation and purification of an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. *Enzyme and microbial technology*, 33(5), 720-724.

Rüdiger, A., Jorgensen, P. L., & Antranikian, G. (1995). Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. *Applied and Environmental microbiology*, 61(2), 567-575.

Ruijssenaars, H., & Hartmans, S. (2001). Plate screening methods for the detection of polysaccharase-producing microorganisms. *Applied Microbiology and Biotechnology*, 55(2), 143-149.

Saha, B. C., Lamed, R., Lee, C. Y., Mathupala, S. P., & Zeikus, J. G. (1990). Characterization of an endo-acting amylopullulanase from *Thermoanaerobacter* strain B6A. Applied and environmental microbiology, 56(4), 881-886.

Sambrook, J., & Russell, D. W. (2001). Molecular cloning: a laboratory manual. third. Cold spring Harbor Laboratory Press, New York.

Sarwar, G., Matayoshi, S., & Oda, H. (1987). Purification of a κ-carrageenase from marine *Cytophaga* species. *Microbiology and immunology*, 31(9), 869-877.

Sata, H., Umeda, M., Kim, C. H., Taniguchi, H., & Maruyama, Y. (1989). Amylase-pullulanase enzyme produced by *B. circulans* F-2. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 991(3), 388-394.

Sawabe, T., Ezura, Y., & Kimura, T. (1992) Purification and characterization of an alginate lyase from marine *Alteromonas* sp. *Nippon Suisan Gakkaishi* 58(3), 521–527

Schiller, N. L., Monday, S. R., Boyd, C. M., Keen, N. T., & Ohman, D. E. (1993). Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (algL): cloning, sequencing, and expression in *Escherichia coli*. *Journal of bacteriology*, 175(15), 4780-4789.

Schultz-Johansen, M., Glaring, M. A., Bech, P. K., & Stougaard, P. (2016). Draft genome sequence of a novel marine bacterium, *Paraglaciecola* sp. strain S66, with hydrolytic activity against seaweed polysaccharides. *Genome announcements*, 4(2), e00304-16.

Shen, G. J., Srivastava, K. C., Saha, B. C., & Zeikus, J. G. (1990). Physiological and enzymatic characterization of a novel pullulan-degrading thermophilic *Bacillus* strain 3183. *Applied microbiology and biotechnology*, 33(3), 340-344.

Shoseyov, O., Shani, Z., & Levy, I. (2006). Carbohydrate binding modules: biochemical properties and novel applications. *Microbiology and molecular biology reviews*, 70(2), 283-295.

Solbak, A. I., Richardson, T. H., McCann, R. T., Kline, K. A., Bartnek, F., Tomlinson, G., ... & Luginbühl, P. (2005). Discovery of pectin-degrading enzymes and directed

evolution of a novel pectate lyase for processing cotton fabric. *Journal of Biological Chemistry*, 280(10), 9431-9438.

Soto-Gil, R. W. (1984). Cloning of *Vibrio harveyi* chitinase and chitobiase genes. *Escherichia coli* in *Chitin, Chitosan, and Related Enzymes*, 209-223.

Spreinat, A., & Antranikian, G. (1990). Purification and properties of a thermostable pullulanase from *Clostridium thermosulfurogenes* EM1 which hydrolyses both α-1, 6 and α-1, 4-glycosidic linkages. *Applied Microbiology and Biotechnology*, 33(5), 511-518.

Stevens, R. A., & Levin, R. E. (1977). Purification and characteristics of an alginase from *Alginovibrio aquatilis*. *Applied and environmental microbiology*, 33(5), 1156-1161.

Sugano, Y., Matsumoto, T., & Noma, M. (1994). Sequence analysis of the agaB gene encoding a new β-agarase from *Vibrio* sp. strain JT0107. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1218(1), 105-108.

Sugano, Y., Matsumoto, T., Kodama, H., & Noma, M. (1993). Cloning and sequencing of agaA, a unique agarase 0107 gene from a marine bacterium, *Vibrio* sp. strain JT0107. *Applied and environmental microbiology*, 59(11), 3750-3756.

Sugano, Y., Terada, I., Arita, M., Noma, M., & Matsumoto, T. (1993). Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. *Applied and Environmental Microbiology*, 59(5), 1549-1554.

Sun, C., Chen, Y. J., Zhang, X. Q., Pan, J., Cheng, H., & Wu, M. (2014). Draft genome sequence of *Microbulbifer elongatus* strain HZ11, a brown seaweed-degrading bacterium with potential ability to produce bioethanol from alginate. *Marine genomics*, *18*, 83-85.

Sun, C., Fu, G. Y., Zhang, C. Y., Hu, J., Xu, L., Wang, R. J., ... & Zhang, X. Q. (2016). Isolation and complete genome sequence of *Algibacter alginolytica* sp. nov., a novel seaweed-degrading Bacteroidetes bacterium with diverse putative polysaccharide utilization loci. *Applied and environmental microbiology*, 82(10), 2975-2987.

Sun, F., Ma, Y., Wang, Y., & Liu, Q. (2010). Purification and characterization of novel κ-carrageenase from marine *Tamlana* sp. HC4. *Chinese Journal of Oceanology and Limnology*, *28*(6), 1139-1145.

Sutherland, I. W., & Keen, G. A. (1981). Alginases from *Beneckea pelagia* and *Pseudomonas* spp. *J. appl. Biochem*., 3(1), 48-57.

Suzuki, H., Sawai, Y., Suzuki, T., & Kawai, K. (2002). Purification and characterization of an extracellular α-neoagarooligosaccharide hydrolase from *Bacillus* sp. MK03. *Journal of bioscience and bioengineering*, 93(5), 456-463.

Suzuki, Y., Hatagaki, K., & Oda, H. (1991). A hyperthermostable pullulanase produced by an extreme thermophile, *Bacillus flavocaldarius* KP 1228, and evidence for the proline theory of increasing protein thermostability. *Applied microbiology and biotechnology*, 34(6), 707-714.

Svitil, A. L., Chadhain, S. M. N. R., Moore, J. A., & Kirchman, D. L. (1997). Chitin Degradation Proteins Produced by the Marine Bacterium *Vibrio harveyi* Growing on Different Forms of Chitin. *Applied and Environmental Microbiology*, 63(2), 408-413.

Swift, S. M., Hudgens, J. W., Heselpoth, R. D., Bales, P. M., & Nelson, D. C. (2014). Characterization of AlgMsp, an alginate lyase from *Microbulbifer* sp. 6532A. PloS one, 9(11), e112939.

Takasaki, Y. (1987). Pullulanase-amylase complex enzyme from *Bacillus subtilis*. *Agricultural and biological chemistry*, 51(1), 9-16.

Tanaka, T., Yan, L., & Burgess, J. G. (2003). *Microbulbifer arenaceous* sp. nov., a new endolithic bacterium isolated from the inside of red sandstone. *Current microbiology*, 47(5), 412-416.

Teeri, T. T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., & Jones, T. A. (1998). *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose?. *Biochem. Soc. Trans*. 26:173-178.

Temuujin, U., Chi, W. J., Lee, S. Y., Chang, Y. K., & Hong, S. K. (2011). Overexpression and biochemical characterization of DagA from *Streptomyces coelicolor* A3 (2): an endo-type β-agarase producing neoagarotetraose and neoagarohexaose. *Applied microbiology and biotechnology*, 92(4), 749-759.

Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., & Dijkstra, B. W. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and x-ray structure of a complex with allosamidin evidence for substrate assisted catalysis. *Biochemistry*, 34(48), 15619-15623.

Tomme, P., Tilbeurgh, H., Pettersson, G., Damme, J., Vandekerckhove, J., Knowles, J., ... & Claeyssens, M. (1988). Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. *The FEBS Journal*, 170(3), 575-581.

Tomme, P., Warren, R.A., Miller, R.C., Jr., Kilburn, D.G. & Gilkes, N.R. (1995) in Enzymatic Degradation of Insoluble Polysaccharides (Saddler, J.N. & Penner, M., eds.), Cellulose-binding domains: classification and properties. pp. 142-163, *American Chemical Society*, Washington.

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., ... & Inamori, Y. (1993). Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Altermonas* sp. strain O-7. *Journal of bacteriology*, 175(1), 176-181.

Turkenburg, J. P., Brzozowski, A. M., Svendsen, A., Borchert, T. V., Davies, G. J., & Wilson, K. S. (2009). Structure of a pullulanase from *Bacillus acidopullulyticus*. *Proteins: Structure, Function, and Bioinformatics*, 76(2), 516-519.

Uchimura, K., Miyazaki, M., Nogi, Y., Kobayashi, T., & Horikoshi, K. (2010). Cloning and sequencing of alginate lyase genes from deep-sea strains of *Vibrio* and *Agarivorans* and characterization of a new *Vibrio* enzyme. *Marine biotechnology*, 12(5), 526-533.

Van Der Maarel, M. J., Van Der Veen, B., Uitdehaag, J. C., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of biotechnology*, 94(2), 137-155.

Vashist, P., Nogi, Y., Ghadi, S. C., Verma, P., & Shouche, Y. S. (2013). *Microbulbifer mangrovi* sp. nov., a polysaccharide-degrading bacterium isolated from an Indian mangrove. *International journal of systematic and evolutionary microbiology*, *63*(7), 2532-2537.

Vera, J., Alvarez, R., Murano, E., Slebe, J. C., & Leon, O. (1998). Identification of a Marine Agarolytic *Pseudoalteromonas* Isolate and Characterization of Its Extracellular Agarase. *Applied and Environmental Microbiology*, 64(11), 4378-4383.

Vijayaraghavan R, Prabha VK, Rajendran S (2012). Isolation and characterization of K-carrageenase from *Bacillus subtilis*. *World of Science and Technology* 2(9):13-18

Vijayaraghavan, R., & Rajendran, S. (2012). Identification of a novel agarolytic γ-Proteobacterium *Microbulbifer maritimus* and characterization of its agarase. *Journal of basic microbiology*, 52(6), 705-712.

Vishnu, C., Naveena, B. J., Altaf, M. D., Venkateshwar, M., & Reddy, G. (2006). Amylopullulanase—A novel enzyme of L. amylophilus GV6 in direct fermentation of starch to L (+) lactic acid. *Enzyme and Microbial Technology*, 38(3), 545-550.

Vorwerg, W., Radosta, S., & Leibnitz, E. (2002). Study of a preparative-scale process for the production of amylose. *Carbohydrate Polymers*, 47(2), 181-189.

Wang, J., Jiang, X., Mou, H., & Guan, H. (2004). Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. *Journal of Applied Phycology*, 16(5), 333-340.

Wang, J., Mou, H., Jiang, X., & Guan, H. (2006). Characterization of a novel β-agarase from marine *Alteromonas* sp. SY37–12 and its degrading products. *Applied microbiology and biotechnology*, 71(6), 833.

Wang, L., Li, S., Yu, W., & Gong, Q. (2015). Cloning, overexpression and characterization of a new oligoalginate lyase from a marine bacterium, *Shewanella* sp. *Biotechnology letters*, 37(3), 665-671.

Wang, S. L., & Chang, W. T. (1997). Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Applied and Environmental Microbiology*, 63(2), 380-386.

Wang, Y. H., Yu, G. L., Wang, X. M., Lv, Z. H., Zhao, X., Wu, Z. H., & Ji, W. S. (2006). Purification and characterization of alginate lyase from marine *Vibrio* sp. YWA. *Acta biochimica et biophysica Sinica*, 38(9), 633-638.

Wang, Y., Guo, E. W., Yu, W. G., & Han, F. (2013). Purification and characterization of a new alginate lyase from a marine bacterium *Vibrio* sp. *Biotechnology letters*, *35*(5), 703-708.

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N. S., Kim, P. B., ... & Lakshmanaswamy, A. (2012). An engineered microbial platform for direct biofuel production from brown macroalgae. *Science*, 335(6066), 308-313.

Watanabe, T. A. K. E. S. H. I., Oyanagi, W., Suzuki, K., Ohnishi, K., & Tanaka, H. (1992). Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *Journal of bacteriology*, 174(2), 408-414.

Wei, W., Ma, J., Chen, S. Q., Cai, X. H., & Wei, D. Z. (2015). A novel cold-adapted type I pullulanase of *Paenibacillus polymyxa* Nws-pp2: in vivo functional expression and biochemical characterization of glucans hydrolyzates analysis. *BMC biotechnology*, 15(1), 96.

Wei, W., Ma, J., Guo, S., & Wei, D. Z. (2014). A type I pullulanase of *Bacillus cereus* Nws-bc5 screening from stinky tofu brine: functional expression in *Escherichia coli* and *Bacillus subtilis* and enzyme characterization. *Process Biochemistry*, 49(11), 1893-1902.

Weiner, R. M., Taylor II, L. E., Henrissat, B., Hauser, L., Land, M., Coutinho, P. M., ... & Bayer, E. A. (2008). Complete genome sequence of the complex carbohydrate-degrading marine bacterium, *Saccharophagus degradans* strain 2-40^{T}. *PLoS genetics*, *4*(5), e1000087.

Wortman, A. T., Somerville, C. C., & Colwell, R. R. (1986). Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. *Applied and environmental microbiology*, 52(1), 142-145.

Wu, H., Yu, X., Chen, L., & Wu, G. (2014). Cloning, overexpression and characterization of a thermostable pullulanase from *Thermus thermophilus* HB27. Protein expression and purification, 95, 22-27.

Wu, S. C., Wen, T. N., & Pan, C. L. (2005). Algal-oligosaccharide-lysates prepared by two bacterial agarases stepwise hydrolyzed and their anti-oxidative properties. *Fisheries Science*, 71(5), 1149-1159.

Xu, J., Ren, F., Huang, C. H., Zheng, Y., Zhen, J., Sun, H., ... & Guo, R. T. (2014). Functional and structural studies of pullulanase from *Anoxybacillus* sp. LM18-11. *Proteins: Structure, Function, and Bioinformatics*, 82(9), 1685-1693.

Yang, J. I., Chen, L. C., Shih, Y. Y., Hsieh, C., Chen, C. Y., Chen, W. M., & Chen, C. C. (2011). Cloning and characterization of β-agarase AgaYT from *Flammeovirga yaeyamensis* strain YT. *Journal of bioscience and bioengineering*, 112(3), 225-232.

Yang, M., Yu, Y., Jin, T., Mou, H., & Li, L. (2017). Genomic analysis of *Microbulbifer* sp. Q7 exhibiting degradation activity toward seaweed polysaccharides. *Marine Genomics*. https://doi.org/10.1016/j.margen.2017.07.003

Yang, S., Yan, Q., Bao, Q., Liu, J., & Jiang, Z. (2017). Expression and biochemical characterization of a novel type I pullulanase from *Bacillus megaterium*. Biotechnology letters, 39(3), 397-405.

Yoon, J. H., Kim, H., Kang, K. H., Oh, T. K., & Park, Y. H. (2003). Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. *International journal of systematic and evolutionary microbiology*, 53(5), 1357-1361.

Zareian, S., Khajeh, K., Ranjbar, B., Dabirmanesh, B., Ghollasi, M., & Mollania, N. (2010). Purification and characterization of a novel amylopullulanase that converts pullulan to glucose, maltose, and maltotriose and starch to glucose and maltose. *Enzyme and Microbial Technology*, 46(2), 57-63.

Zeng, C., Zhang, L., Miao, S., Zhang, Y., Zeng, S., & Zheng, B. (2016). Preliminary characterization of a novel β-agarase from *Thalassospira profundimonas*. *Springer Plus*, 5(1), 1-8.

Zhang, W. W., & Sun, L. (2007). Cloning, characterization, and molecular application of a beta-agarase gene from *Vibrio* sp. strain V134. *Applied and environmental microbiology*, 73(9), 2825-2831.

Zhang, Z., Yu, G., Guan, H., Zhao, X., Du, Y., & Jiang, X. (2004). Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from *Vibro* sp. 510. *Carbohydrate Research*, 339(8), 1475-1481.

Zhou, M. H., Ma, J. S., Li, J., Ye, H. R., Huang, K. X., & Zhao, X. W. (2008). A κ-carrageenase from a newly isolated *Pseudoalteromonas*-like bacterium, WZUC10. *Biotechnology and Bioprocess Engineering*, *13*(5), 545-551.

Zhu, B. W., Tan, H. D., Qin, Y. Q., Du, Y. G., & Yin, H. (2015). Characterization of a new endo-type polyM-specific alginate lyase from *Pseudomonas* sp. *Biotechnology letters*, 37(2), 409-415.

Zhu, Y., Chen, P., Bao, Y., Men, Y., Zeng, Y., Yang, J., ... & Sun, Y. (2016). Complete genome sequence and transcriptomic analysis of a novel marine strain *Bacillus weihaiensis* reveals the mechanism of brown algae degradation. *Scientific reports*, 6, 38248.

Zhu, Y., Zhao, R., Xiao, A., Li, L., Jiang, Z., Chen, F., & Ni, H. (2016). Characterization of an alkaline β-agarase from *Stenotrophomonas* sp. NTa and the enzymatic hydrolysates. *International journal of biological macromolecules*, 86, 525-534.

Appendix

Artificial Seawater (ASW)

Components	g/L
Tris base	6.05
$MgSO_4$	12.32
KCl	0.74
$(NH)_2\ HPO_4$	0.13
NaC1	17.52
$CaC1_2$	0.14

Above components dissolve in 900 ml of distilled water and pH adjusted to 7.0 with conc. HCI immediately. Volume was make up to 1000 m1 with distilled water before autoclaving.

TE Buffer (pH 8.0)

Components	Quantity
Tris-C1 (10mM)	0.157 g
EDTA (1mM)	0.028 g

Dissolve in 90 ml of water and adjust the pH to 8.0. Make up the volume to 100 ml.

3M Sodium Acetate (pH 5.2)

Components	Quantity
Sodium acetate	40.81 g
Distilled water	80.00 ml

Adjust the pH to 5.2 with acetic acid and make up the volume to 100 ml. Sterilize by autoclaving.

5X TBE buffer

Components	Quantity
Tris Base	54.00 g
Boric acid	27.50 g
0.5 M EDTA	20.00 ml

Dissolve 186.1 g of di sodium EDTA in 800 ml of water and adjust the pH to 8.0 with 1M NaOH) Dissolve in 800 ml of water and make up the volume to 1000 ml.

12% SDS-PAGE Composition

Components	Volume (ml)
12 % Resolving gel (5 ml)	
Milli Q water	1.6
30 % Acrylamide Mix	2.5
1.5 M Tris (pH 8.8)	1.3
10% SDS :	0.05
10% Ammonium persulphate	0.05
TEMED	0.002
5% Stacking gel (3 ml)	
Milli Q water	2.1
30 % Acrylamide Mix	0.5
1.5 M Tris (pH 8.8)	0.38
10% SDS :	0.03
10% Ammonium persulphate	0.03
TEMED	0.003

6X SDS-PAGE loading buffer

Components	Quantity
4X Tris-CI (pH 6.8)	7.0 ml
Glycerol	3.0 ml
SDS	1.0 g
DTT	0.93 g
Bromophenol blue	1.2 g

6X Native PAGE loading buffer

Components	Quantity
4X Tris-Cl(pH 6.8) :	7 ml
Glycerol :	3 ml
Bromophenol blue	1.2 g

Coomassie Brilliant Blue stain

Components	Quantity
Coomassie Brilliant Blue R-250	0.25 g
Methanol	50 ml
Acetic acid (glacial)	10 ml
Distilled water	40 ml

Publications

A) Published articles

1. **Imran, Md**., Pant, P., Shanbhag, Y. P., Sawant, S. V., & Ghadi, S. C. (2017). Genome Sequence of *Microbulbifer mangrovi* DD-13^{T} Reveals Its Versatility to Degrade Multiple Polysaccharides. *Marine Biotechnology*, *19*(1), 116-124.

2. **Imran, Md**., Poduval, P. B., & Ghadi, S. C. (2017). Bacterial Degradation of Algal Polysaccharides in Marine Ecosystem. In *Marine Pollution and Microbial Remediation* (pp. 189-203). Springer Singapore.

3. **Imran, Md**., Saida, B., Ghadi, S. C., Verma, P., & Shouche, Y. S. (2016). The gut-associated *Klebsiella* sp. of the apple snail produces multiple polysaccharide degrading enzymes. *Current Science*, 110(11), 2170-2172.

B) Manuscripts to be communicated

1. **Imran, Md**., & Ghadi, S. C., Cloning and sequencing of gene encoding type-I pullulanase from *Microbulbifer mangrovi* DD-13^{T} and characterization of gene product

2. **Imran, Md**., & Ghadi, S. C., Characteristics of polysaccharide-degrading genes of *Microbulbifer mangrovi* DD-13^{T}

C) Abstract published in conferences

1. **Imran, Md.,** & Ghadi, S. C., "Carbohydrate active enzymes of *Microbulbifer mangrovi* DD-13^{T}: An insight from genome sequence", National conference of young researchers on new frontiers in life science and environment, Goa University, Goa. Mar 16-17, 2017

2. **Imran, Md.,** & Ghadi S. C., "Preparation of a genomic library from multiple polysaccharides degrading *Pseudomonas* sp. and screening for agar degradation, National seminar on Life and life processes: Sustainable development, Dept. of Zoology, Goa University, Goa. Feb 19-21, 2015

3. **Imran, Md.**, Poduval, P. B., & Ghadi, S. C. "Cloning of esterase gene from a bacterial strain CR-1", 55th Annual conference of Association of Microbiologists of India (AMI) on "Empowering Mankind with Microbial Technologies" at Tamil Nadu Agricultiral University, Coimbatore, Tamil Nadu, Nov. 12-14, 2014

4. **Imran, Md.,** & Ghadi, S. C., "Cloning of agarse gene from a multiple polysaccharide degrading bacteria *Microbulbifer* strain CMC-5", in DBT-JRF regional meet at Institute of Chemical Technology (ICT), Matunga, Mumbai. Nov. 21-22, 2013