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ORIGINAL ARTICLE

Genome Sequence of *Microbulbifer mangrovi* DD-13^T Reveals Its Versatility to Degrade Multiple Polysaccharides

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Abstract *Microbulbifer mangrovi* strain DD-13^T is a novel-type species isolated from the mangroves of Goa, India. The draft genome sequence of strain DD-13 comprised 4,528,106 bp with G+C content of 57.15%. Out of 3479 open reading frames, functions for 3488 protein coding sequences were predicted on the basis of similarity with the cluster of orthologous groups. In addition to protein coding sequences, 34 tRNA genes and 3 rRNA genes were detected. Analysis of nucleotide sequence of predicted gene using a Carbohydrate-Active Enzymes (CAZymes) Analysis Toolkit indicates that strain DD-13 encodes a large set of CAZymes including 255 glycoside hydrolases, 76 carbohydrate esterases, 17 polysaccharide lyases, and 113 carbohydrate-binding modules (CBMs). Many genes from strain DD-13 were annotated as carbohydrases specific for degradation of agar, alginate, carrageenan, chitin, xylan, pullulan, cellulose, starch, β-glucan, pectin, etc. Some of polysaccharide-degrading genes were highly modular and were appended at least with one CBM indicating the versatility of strain DD-13 to degrade complex polysaccharides. The cell growth of strain DD-13 was validated 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 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 and were associated with concomitant production of homologous carbohydrase.

Keywords Carbohydrate-binding module · Glycoside hydrolases · *Microbulbifer mangrovi* · Alginate lyase · Agarase

Accession No. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBankunder the accession LZDE00000000. The version described in this paper is version LZDE01000000.

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Introduction

The cell wall of plants and seaweeds is a conglomerate of recalcitrant complex polysaccharides (CPs) fortifying against microbial intrusion and harsh environment. The eventual decay of plants and seaweeds culminates into CP-enriched litter and marine snow that promotes epiphytic association of carbohydrase-degrading bacteria. The recycling of carbon from CPs in the marine ecosystem is widely attributed to versatility of multiple polysaccharide-degrading bacteria such as *Saccharophagus degradans* and *Microbulbifer* species (Hutcheson et al. 2011; Jonnadula et al. 2009; Wakabayashi et al. 2012; Vashist et al. 2013). Besides their innate role in degradation of CP from litter, the multiple polysaccharide-degrading bacteria have already 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).

Carbohydrate-Active Enzymes (CAZymes) are a group of enzymes that are implicated in the breakdown or modification of polysaccharides. Genome sequencing and functional annotation enable a comprehensive identification of CAZymes facilitating a holistic understanding of their ecological role and exploiting them for creating novel technologies. Recently, genomes of several CP-degrading bacteria have been sequenced with emphasis on annotation of CAZyme genes. The *Saccharophagus degradans* genome has been completely sequenced, and the CAZyme genes have been elaborately analyzed (Weiner et al. 2008). Additionally, the draft genome sequence of CP-degrading bacteria such as *Microbulbifer* strain HZ11 (Sun et al. 2014), *Flammeovirga* sp. OC4 (Liu et al. 2015), *Alteromonadaceae* sp. strain G7 (Kwak et al. 2012), *Bacillus niacin* strain JAM F8 (Kurata et al. 2014), *Vibrio* sp. strain EJY3 (Roh et al. 2012), and *Formosa agariphila* KMM 3901(Mann et al. 2013) have been reported.

Microbulbifer mangrovi strain DD-13 is a novel-type species isolated from the mangroves of Goa, India, with the capability of degrading a diverse range of polysaccharides (Vashist et al. 2013). In the present study, general characteristics of a draft genome sequence of strain DD-13 are reported. The genome annotation of strain DD-13 revealed diverse CAZymes in comparison to other CP-degrading bacteria that empower the former to degrade various polysaccharides that are constituents of plant and algal cell wall. Additionally, strain DD-13 was demonstrated to produce carbohydrase that degraded agar and alginate with concomitant release of reducing sugar as metabolizable carbon sugar. Further, seaweed powder prepared from dried red and brown seaweeds also promoted growth of strain DD-13 endorsing the genome annotation data with regards to its potential to degrade plant and algal wastes.

Methodology

Growth Condition and Genomic DNA Isolation From *Microbulbifer mangrovi* Strain DD-13

A single colony of bacterial strain DD-13 was inoculated in Zobell Marine Broth (HiMedia, Mumbai, India) and grown for 24 h at 30 °C on an orbital shaker. The genomic DNA was isolated using a 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).

Genome Sequencing and Annotation

For next generation sequencing, a genomic library was prepared from strain DD-13 genomic DNA. An Agilent SureSelect adapter was added to the DNA fragments, and the sequence was read on an Illumina NextSeq 500 platform. A total of 6,862,280 reads with an average read length of 151 bp were obtained. 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 "N" 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).

Identification and Analysis of Polysaccharide-Degrading Genes

The result of the BLAST search of all predicted genes was imported in 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 and individually analyzed by blastx and blastp against NCBI nr-protein database.

CAZyme Analysis

The open reading frame (ORF) predicted in Glimmer was analyzed against CAZy database (Lombard et al. 2014). Briefly, the sequence file of the predicted ORF 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.

Degradation of Agar and Alginate by *Microbulbifer* Strain DD-13

The starter culture was prepared by inoculating a loopful of strain DD-13 culture in an artificial seawater (ASW) medium (g L^{-1}; Tris base 6.05, $MgSO_4 \cdot 7H_2O$ 12.32, KCL 0.74, $(NH_4)_2HPO_4$ 0.13, NaCl 17.52, and $CaCl_2$ 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 milliliter 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. Maltose was used as standard. 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.

Degradation of Seaweed

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 form seaweed fine powder that was stored at 4 °C in screw-capped vials.

One milliliter 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.

Bacterial Growth Studies with Other Polysaccharides and Determination of Homologous Carbohydrase Activities

The bacterial strain DD-13 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.

Results and Discussion

Genome Specifics

The genome of DD-13 was sequenced with whole-genome shotgun sequence strategy using the Illumina NextSeq 500 platform. Quality-filtered paired-end reads were assembled using the SOAPdenovo alignment tool (http://soap.genomics.org.cn/soapdenovo.html). The assembly generated a total of 370 longer contigs with the average contig length of 7075 bp. (Table 1). 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 is of 4,528,106 bp with a GC content of 57.15%, and the values are proximate to the reported draft genome size (4,223,108 bp) and GC content (56.70%) of *Microbulbifer elongatus* strain HZ11(Sun et al. 2014). The genome size of strain DD-13 is also comparatively closer to the completely sequenced genome size of *S. degradans* (Weiner et al. 2008). On the contrary, *Flammeovirga* sp. OC4, an algal polysaccharide-degrading bacterium, has a bigger draft genome size (8,065,497 bp) (Liu et al. 2015).

A total of 3749 ORFs were predicted in the genome of strain DD-13. Out of 3749 ORFs, 3488 (93.0%) are protein-coding genes that demonstrated similarity with proteins in the

Table 1 Genome assembly and general features of strain DD-13 genome

Category	Number	% of total
Total number of contigs	370	–
Total number of bases (assembly size)	4,528,106	100
DNA coding number of bases (bp)	4,457,629	98.44
DNA G+C number of bases	2,587,717	57.15
DNA scaffolds	37	–
Total number of genes predicted	3749	100
Protein coding sequences (including hypothetical proteins)	3488	93.03
Genes in COGs	3348	89.3
Total number of RNA genes	37	0.99
rRNA genes		
5S rRNA	1	0.03
16S rRNA	1	0.03
23S rRNA	1	0.03
tRNA genes	34	0.91

NCBI nr-database with an e-value of $<1e^{-5}$. Similarly in *S. degradans*, 4008 protein-encoding genes were predicted and 3795 (94.7%) proteins depicted a match with the nr-database proteins (Weiner et al. 2008). Likewise, 3293 protein-coding genes were predicted from the genome of *M. elongatus* strain HZ11 (Sun et al. 2014). Our analysis indicates that the 3488 candidate genes of strain DD-13 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. Hypothetical proteins are known as novel prospective targets for future experimental studies (Galperin and Koonin 2004), and thus, 30% of predicted genes in strain DD-13 are probable novel genes.

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 were functionally annotated (Table 2).

In addition to protein-encoding genes, 34 tRNA and 3 rRNA genes were also predicted in strain DD-13 (Table 1) in comparison to 41 tRNA and 6 rRNA genes predicted in *S. degradans* (Weiner et al. 2008). Further, the reported tRNA genes from strain DD-13 are fewer than that reported in *Escherichia coli* strains that possess 90–100 tRNA genes whereas *Pseudomonas aeruginosa* strains harbor 55–64 tRNA genes (Weiner et al. 2008).

Table 2 Clusters of Orthologous Groups of proteins functional categories of genes from *Microbulbifer mangrovi strain* DD-13 (Tatusov et al. 2000)

COG class	Total count (percentage)[a]	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, and 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

[a] Percentage has been calculated based on total number of functionally assigned genes (3348)

Table 3 Diverse genes related to polysaccharide degradation identified in the genome of *Microbulbifer mangrovi* strain DD-13

Sr. no.	Annotation of polysaccharide degrading genes	Contig no. and position of gene	CAZy family
1	Agarase	contig1094: 14,877–18,161	CBM6|GH86
		contig1270: 133,540–135,879	GH50
		contig1270: 148,758–151,022	GH50
		contig908: 82–1140	CBM6|GH16
		contig908: 1201–3651	GH86
2	Alginate lyase	contig1246: 14,949–17,225	PL6
		contig1246: 17,247–19,490	PL17
3	Carrageenase	contig1270: 97,064–101,125	GH82
4	Chitinase	contig1176: 1453–4569	CBM5|GH18
		contig1212: 35,745–36,755	GH18
		contig1270: 61,090–62,316	CBM5|GH18
		contig1270: 62,760–65,579	CBM5|GH18
5	Xylanase	contig884: 105–2843	CBM6|CBM22|GH10
		contig1090: 9803–11,326	CBM10|CBM2|GH10
6	Pullulanase	contig1082: 12,386–14,932	GH13
7	Amylase	contig1204: 24,298–26,052	GH13
		contig1230: 35,822–37,561	GH13
		contig1230: 14,935–16,878	GH13
		contig1230: 7994–10,270	GH13
		contig1090: 9803–11,326	CBM10|CBM2|GH10
8	Pectate lyase	contig1240: 21,784–23,556	PL1
		contig1240: 27,163–31,128	PL1|CBM35
		contig1240: 14,289–15,569	CBM35|PL10
9	Glycoside hydrolase	contig1024: 3158–4504	GH28
		contig1116: 17,660–19,456	GH2
		contig1260: 36,351–37,820	–
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: 19,531–20,925	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: 10,922–12,475	GH43
13	Glycoside hydrolase family protein	contig1270: 76,325–77,794	GH16
14	Arylsulfastase	contig1150: 13,661–15,196	CBM51
		contig1150: 15,278–17,521	GH43
		contig1150: 19,821–21,356	CBM51
		contig1270: 73,306–74,748	CBM51
		contig1270: 117,398–118,831	CBM51
15	α-Glucuronidase	contig1066: 5042–7222	GH67
16	Exo-poly-α-D-galacturonosidase	contig1240: 47,262–48,638	GH28
17	Endo-1,4-D-glucanase	contig1218: 31,916–32,779	–
18	β-Glucanase	contig1266: 72,943–74,391	CBM6|GH16
19	1,4 β-D-xylanxylohydrolase	contig1236: 44,604–46,211	GH43
20	Glucan 1,4-α-glucosidase	contig1104: 2594–19,458	GH43
21	β-Mannosidase	contig1036: 3363–4805	GH5|CBM10
22	α-L-fucosidase	contig1158: 23,994–26,477	GH95
23	Cellobiohydrolase	contig1186: 21,070–22,869	GH6|CBM2
24	Putative glucoamylase I	contig1034: 8599–9165	–
25	Xyloglucanase	contig1036: 105–3311	CBM10|CBM2|GH74
26	β-Glucosidase	contig1058: 15–1493	GH3
		contig1102: 14,996–16,333	GH1
		contig1266: 72,943–74,391	CBM6|GH16
27	α-/glucosidase	contig1158: 12,819–14,477	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: 9285–10,262	GH43

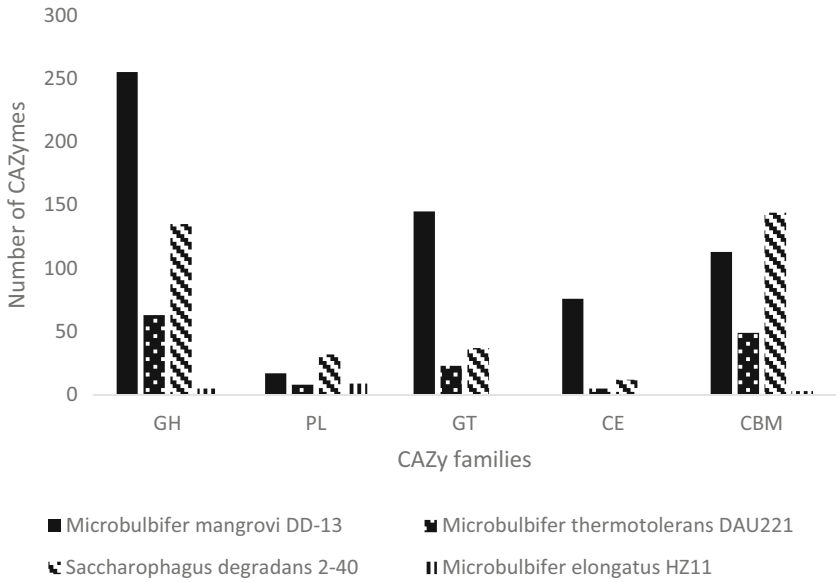

Fig. 1 Comparison of total number of CAZymes present in the genome of different multiple polysaccharide-degrading bacteria, namely, *Microbulbifer mangrovi* DD-13, *Microbulbifer thermotolerans* DAU221, *Saccharophagus degradans* 2–40, and *Microbulbifer elongatus* HZ11 (*GH* glycoside hydrolase, *PL* polysaccharide lyase, *GT* glycosyl transferase, *CE* carbohydrate esterase, *CBM* carbohydrate-binding module) (the information of CAZymes of *M. thermotolerans* DAU221 and *S. degradans* 2–40 were taken from the CAZy database)

Polysaccharide-Degrading Genes

The capability of *M. mangrovi* strain DD-13 to degrade various polysaccharides (Vashist et al. 2013) was evident from the annotation of the DD-13 genome. The genome of DD-13 encodes several proteins capable of degrading a diverse range of algal and plant cell wall polysaccharides such as agar, alginate, carrageenan, chitin, xylan, pullulan, cellulose, glucan, pectin, starch, fucoidan, etc. (Table 3).

Five agar-degrading genes belonging to GH86, GH50, and GH16 families were predicted. In the CAZy database, the members of GH86 and GH50 families are known for β-agarase and porphyranase activity whereas the proteins belonging to the GH16 family demonstrate various enzyme activities including β-agarase activity (Lombard et al. 2014). All members of GH50 are neoagarotetraose-producing agarase suggesting that the two agarase observed in the strain of DD-13 would degrade agar to neoagarotetraose. Additionally, two agarase genes have a CBM6 module that would promote efficient and prolonged binding of enzyme with agar.

Additionally, the two alginate lyases identified in the genome of DD-13 are classified in the PL6 and PL17 families. The known enzyme activities under the PL6 family are alginate lyases (EC 4.2.2.3), chondroitinase (EC 4.2.2.19), and MG-specific alginate lyase (4.2.2.-) whereas the PL17 family is known for the alginate lyase (EC 4.2.2.3) and oligo alginate lyase (EC 4.2.2.-) activities.

The carrageenase gene identified in strain DD-13 belonged to the GH82 family. All members of the GH82 family are reported to demonstrate carrageenase activity against iota carrageenan.

Furthermore, a total of four chitinase genes were identified in strain DD-13. These four chitinase genes are classified as GH18 chitinase. Proteins belonging to family GH18 are candidate chitinases. Out of these four, three chitinases were predicted to be extracellular and are appended with the CBM5 module.

The GH10 family includes the endo-1,4-β-xylanase (EC 3.2.1.8) or/and endo-1,3-β-xylanase (EC 3.2.1.32) suggesting that the two xylanases identified in strain DD-13 are endo-xylanases. These two xylanases are modular enzymes, as CBM6 and CBM22 modules are observed in one of the xylanases while the other has CBM10 and CBM2 modules. The CBM2 module has been known for binding with xylan while CBM10 has been known for binding with cellulose. The modular structure of xylanase promotes the binding of enzyme to the substrate.

Pectin degradation could be achieved by pectin lyase, pectate lyase, and polygalacturonase (Abbott and Boraston 2008). In strain DD-13, 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 in the CAZy database.

Cellulose hydrolysis is generally achieved by the synergistic action of endo-glucanases, exo-glucanases, and β-glucosidases. The DD-13 genome encodes all the required enzymes to carry out the cellulose hydrolysis (Table 3).

Our analysis also reveals that the DD-13 genome encodes five amylases. The four amylases are 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.

Furthermore, several other polysaccharide-degrading genes identified include arylsulfatase, 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,

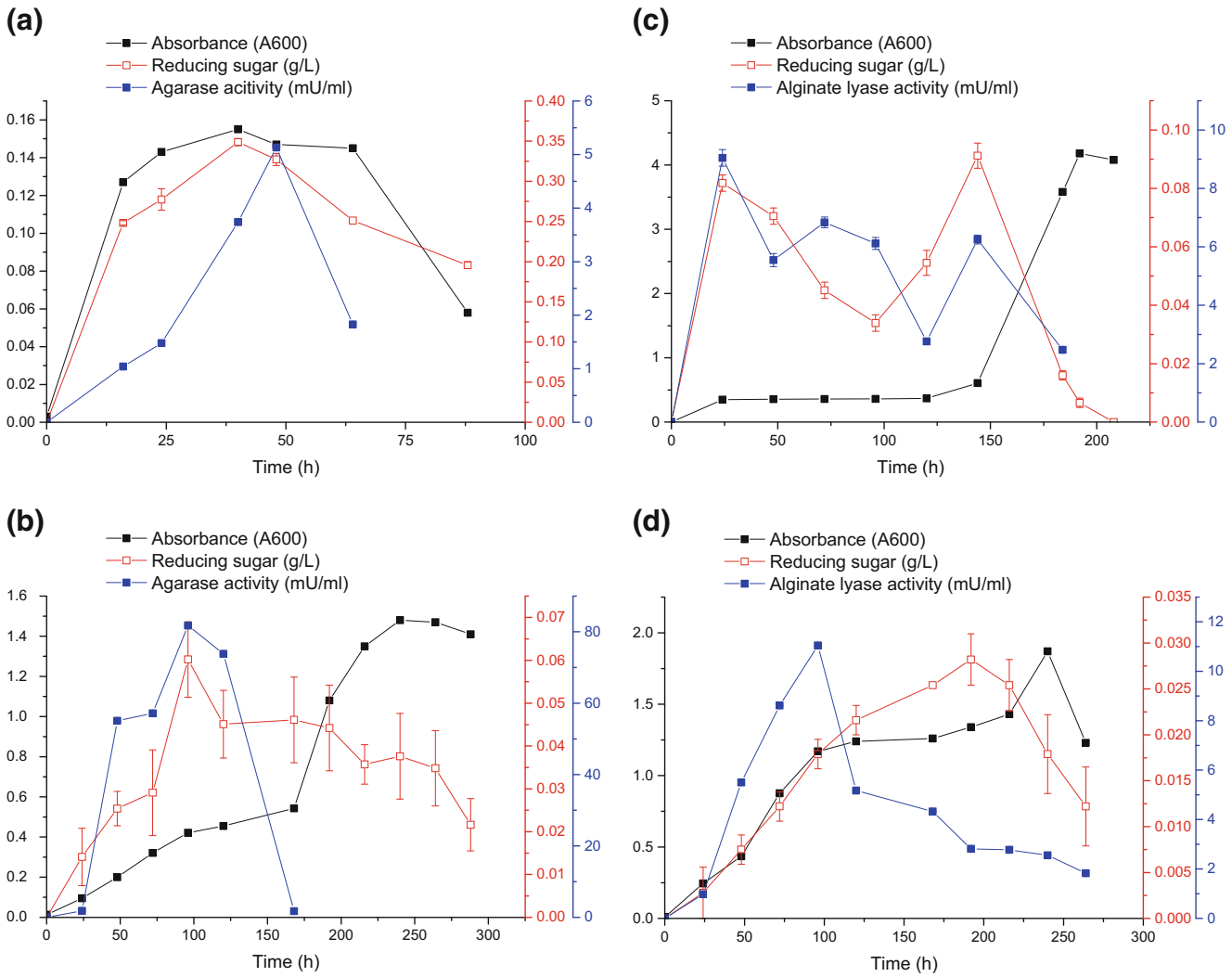

Fig. 2 Profile of cell growth, reducing sugar, and enzyme activity during growth of *Microbulbifer mangrovi* DD-13 in presence of **a** agarose, **b** *Gracilaria* seaweed powder, **c** alginate, and **d** *Sargassum* seaweed powder

putative glucoamylase I, xyloglucanase, β-glucosidase, α-glucosidase, β-glycosidase, arabinanendo-1,5-α-L-arabinosidase, and α-N-arabinofuranosidase (Table 3).

CAZymes of *Microbulbifer mangrovi* Strain DD-13

CAZymes are involved in the metabolism of carbohydrates and glycoconjugates. In the CAZy database, CAZymes are classified into four groups including glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), and carbohydrate esterases (CEs) (Cantarel et al. 2009). Along with the catalytic modules, CAZymes also contain carbohydrate-binding modules (CBMs) that promote the prolonged and efficient binding of enzymes with the target substrate (Cantarel et al. 2009). Microorganisms producing GHs and CBMs are important for the degradation of the complex polysaccharides for biofuel production (Lynd et al. 2005). Thus, these enzymes are of special interest for studies aiming to reduce the difficulty in hydrolyzing biomass in bioenergy crops (McCann and Carpita 2008). Polysaccharide-degrading microorganisms produce a repertoire of glycoside hydrolases which target glycosidic bonds to elicit the degradation of recalcitrant-insoluble polysaccharides. However, glycosidic bonds are often inaccessible for the active site of glycoside hydrolases, and the presence of high-affinity non-catalytic carbohydrate-binding modules assists the enzyme in accessing the target glycosidic bonds and promotes the association of the enzyme with the substrate (Boraston et al. 2004).

As seen in Fig. 1, 255 genes belonging to the different GH family have been identified in the *M. mangrovi* strain DD-13 genome. Additionally, in comparison to other multiple polysaccharide-degrading bacteria, strain DD-13 depicts a wide range of CAZymes (Fig. 1). The most frequent types of GH encoded by DD-13 are GH43 (24), GH109 (19), GH92 (18), GH3 (18), GH23 (16), and GH53 (15) (Supplementary Table 1). A total of 30 GHs are appended with at least 1

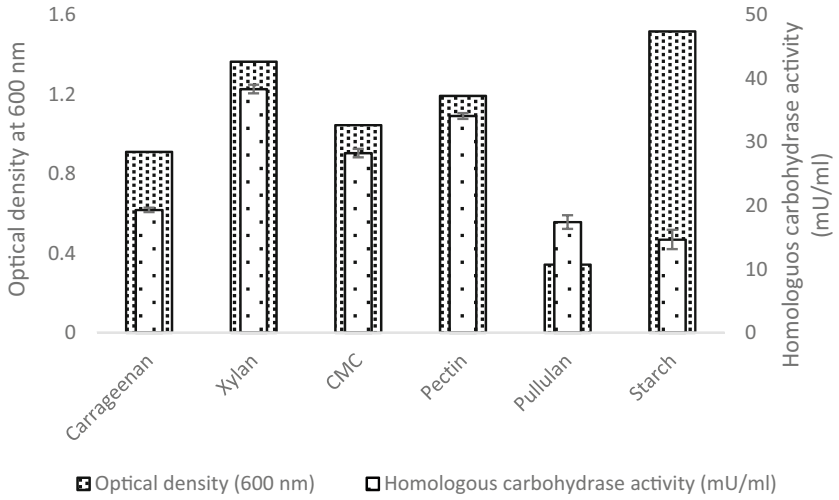

Fig. 3 Growth of strain DD-13 and homologous carbohydrase activity at 24 h in the presence of individual polysaccharides as a sole carbon substrate

CBM including 7 GHs which are appended to more than 1 CBM (Table 3). GHs that appended to more than one CBM demonstrate very strong affinity for substrate polysaccharides through avidity effects (Freelove et al. 2001; Boraston et al. 2002). In addition to GHs, the DD-13 genome encodes a large number of CBMs (113). Only the *Saccharophagus* genome encodes more CBMs (127) than the DD-13 genome while *Arabidopsis thaliana*, the fungus *Magnaporthe grisea*, and *Homo sapiens* encode 92, 66, and 35 CBMs respectively (Weiner et al. 2008). The genome of DD-13 has frequent numbers of CBM50 (19), CBM2 (17), and CBM32 (13).

Degradation of Polysaccharides and Seaweed

Strain DD-13 degraded agarose assisted by extracellular agarase leading to the production of reducing sugars. As observed from Fig. 2a, 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 to degrade seaweed. As seen from Fig. 2b, 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 which is higher than that reported from *Bacillus* sp. SYR4 (Kang and Kim 2015).

Strain DD-13 also degraded alginate with concomitant production of extracellular alginate lyase leading to production of reducing sugar. As seen in Fig. 2c, 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. 2d, strain DD-13 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 which is closer to values reported for *Microbacterium oxydans* (Kim et al. 2013).

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. 3). 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. The production and determination of carbohydrase activities from strain DD-13 during its growth in respective polysaccharides support the genomic data and indicate the true potential of strain DD-13 to degrade various complex polysaccharide degradations. Further, using dye-based plate qualitative assay, bacterial strain DD-13 has already been reported to degrade 11 polysaccharides including chitin, laminarin, and β-glucan (Vashist et al. 2013).

The genome sequence of *M. mangrovi* DD-13 reveals extraordinary potential for degradation of multiple polysaccharides. The genome harbors 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. Cloning and expression of the carbohydrase from strain DD-13 are currently under progress.

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References

Abbott DW, Boraston AB (2008) Structural biology of pectin degradation by Enterobacteriaceae. Microbiol Mol Biol Rev 72:301–316

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382:769–781

Boraston AB, McLean BW, Chen G, Li A, Warren RA, Kilburn DG (2002) Cooperative binding of triplicate carbohydrate-binding modules from a thermophilic xylanase. Mol Microbiol 43:87–194

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:D233–D238

Delcher AL, Harmon D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27:4636–4641

Freelove AC, Bolam DN, White P, Hazlewood GP, Gilbert HJ (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromycesequi. J Biol Chem 276: 43010–43017

Galperin MY, Koonin EV (2004) 'Conserved hypothetical' proteins: prioritization of targets for experimental study. Nucleic Acids Res 32: 5452–5463

Hutcheson SW, Zhang H, Suvorov M (2011) Carbohydrase systems of *Saccharophagus degradans* degrading marine complex polysaccharides. Mar drugs 9:645–665

Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119

Jonnadula R, Verma P, Shouche YS, Ghadi SC (2009) Characterization of *Microbulbifer* strain CMC-5, a new biochemical variant of *Microbulbifer elongatus* type strain DSM6810^T isolated from decomposing seaweeds. Curr Microbiol 59:600–607

Kang S, Kim JK (2015) Reuse of red seaweed waste by a novel bacterium, *Bacillus* sp. SYR4 isolated from a sandbar. World J Microbiol Biotechnol 31:209–217

Kim EJ, Fathoni A, Jeong GT, Do Jeong H, Nam TJ, Kong IS, Kim JK (2013) *Microbacteriumoxydans*, a novel alginate-and laminarin-degrading bacterium for the reutilization of brown-seaweed waste. J Environ Manag 130:153–159

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 Announc 2:e00983–e00914

Kwak MJ, Song JY, Kim BK, Chi WJ, Kwon SK, Choi S, Kim JF (2012) Genome sequence of the agar-degrading marine bacterium *Alteromonadaceae* sp. strain G7. J Bacteriol 194:6961–6962

Lagesen K, Hallin P, Rødland EA, Stærfeldt HH, Rognes T, Ussery DW (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35:3100–3108

Liu Y, Yi Z, Cai Y, Zeng R (2015) Draft genome sequence of algal polysaccharides degradation bacterium, *Flammeovirga* sp. OC4. Mar Genomics 21:21–22

Lombard V, Golaconda RH, Drula E, Coutinho PM, Henrissat B (2014) The carbohydrate active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495

Lowe TM, Eddy SR (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25:955–964

Lynd LR, Van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583

Mann AJ, Hahnke RL, Huang S, Werner J, Xing P, Barbeyron T, Glöckner FO (2013) The genome of the alga-associated marine flavobacterium *Formosa agariphila* KMM 3901^T reveals a broad potential for degradation of algal polysaccharides. Appl Environ Microbiol 79:6813–6822

McCann MC, Carpita NC (2008) Designing the deconstruction of plant cell walls. Curr Opin Biotechnol 11:314–320

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153:375–380

Park BH, Karpinets TV, Syed MH, Leuze MR, Uberbacher EC (2010) CAZymes Analysis Toolkit (CAT): web service for searching and analyzing carbohydrate-active enzymes in a newly sequenced organism using CAZy database. Glycobiology 20:1574–1584

Roh H, Yun EJ, Lee S, Ko HJ, Kim S, Kim BY, Choi IG (2012) Genome sequence of *Vibrio* sp. strain EJY3, an agarolytic marine bacterium metabolizing 3, 6-anhydro-L-galactose as a sole carbon source. J Bacteriol 194:2773–2774

Sun C, Chen YJ, Zhang XQ, 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. Mar Genomics 18:83–85

Tatusov RL, Galperin MY, Natale DA, Koonin EV (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28(1):33–6

Tatusov RL, Natale DA, Garkavtsev IV, Tatusova TA, Shankavaram UT, Rao BS, Koonin EV (2001) The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res 29:22–28

Vashist P, Nogi Y, Ghadi SC, Verma P, Shouche YS (2013) *Microbulbifer mangrovi* sp. nov., a polysaccharide-degrading bacterium isolated from an Indian mangrove. Int J Syst Evol Microbiol 63:2532–2537

Wakabayashi M, Sakatoku A, Noda F, Noda M, Tanaka D, Nakamura S (2012) Isolation and characterization of *Microbulbifer* species 6532A degrading seaweed thalli to single cell detritus particles. Biodegradation 23:93–105

Wargacki AJ, Leonard E, Win MN, Regitsky DD, Santos CNS, Kim PB, Lakshmanaswamy A (2012) An engineered microbial platform for direct biofuel production from brown macroalgae. Science 335:308–313

Weiner RM, Taylor LE II, Henrissat B, Hauser L, Land M, Coutinho PM, Bayer EA (2008) Complete genome sequence of the complex carbohydrate-degrading marine bacterium, *Saccharophagus degradans* strain 2-40 T. PLoS Genet 4:e1000087