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Short Communication

Effect of polysaccharide admixtures on expression of multiple polysaccharide-degrading enzymes in *Microbulbifer* strain CMC-5

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ABSTRACT

Microbulbifer strain CMC-5 produces agarase, alginate lyase, xylanase, carboxymethyl cellulase and carrageenase. The extracellular production of the above carbohydrases was investigated by growing *Microbulbifer* strain CMC-5 in a sea water based medium containing homologous/heterologous polysaccharides as a single substrate or as a combination of mixed assorted substrate. Presence of singular homologous polysaccharides in the growth medium induces respective carbohydrase at high levels. Any two polysaccharides in various combinations produced high level of homologous carbohydrase and low level of other heterologous carbohydrase. All five carbohydrases were consistently produced by strain CMC-5, when carboxymethyl cellulose was included as one of the substrate in dual substrate combination, or in presence of mix blends of all five polysaccharides. Interestingly, thalli of *Gracilaria* sp. that contain agar and cellulose predominantly in their cell wall induces only agarase expression in strain CMC-5.

1. Introduction

Insoluble complex polysaccharides (ICPs) such as agar, alginate, carrageenan, xylan and chitin are primarily responsible for maintaining structural integrity in marine organisms. Occasionally, ICPs are combined in heterogeneous proportions to generate a complex recalcitrant polysaccharide framework that is difficult to degrade. Likewise, the cell wall of *Gracilaria* sp. consist of an agarose matrix embedded in a mesh of cellulose network [1,2]. Multiple polysaccharide degrading marine bacteria from decomposing sea grasses and seaweeds have been isolated and participate in recycling of carbon from ICPs [3–6].

In marine ecosystems, the ICPs degrading bacteria would be concurrently exposed to multiple polysaccharides. Although, production of homologous polysaccharide degrading enzymes in presence of respective individual polysaccharides such as cellulose, xylan, agar, alginate and carrageenan have been studied, [7–11] the effect of these individual or mixed polysaccharides on expression of homologous and other heterologous polysaccharide degrading enzymes (carbohydrases) have not been extensively studied. The only other reported studies demonstrating expression of heterologous carbohydrases in presence of individual polysaccharides was from *Saccharophagus degradans* 2–40, a multiple polysaccharide degrader [12].

Microbulbifer strain CMC-5 isolated previously from decomposing seaweeds and degrading multiple polysaccharides was used in the present study [6]. The objective was to determine the expression of different carbohydrases when cellulose or agar or carrageenan or xylan or alginate was provided as single or as a combination of mixed assorted substrates. Additionally, expression of carbohydrases in strain CMC-5 was also studied using an ecological simulation by providing seaweed thalli (*Gracilaria* sp.) as a biomass whose cell wall naturally consists of agar blended with cellulose.

2. Material and methods

2.1. Growth condition

Starter culture of *Microbulbifer* strain CMC-5 (MTCC 9889) was prepared by growing in artificial sea water (ASW) medium [13] containing 0.2% of single or dual polysaccharides or polysaccharides mix containing all the five polysaccharides as carbon substrate and supplemented with 0.05% yeast extract. The polysaccharides used in present study were low melting point (LMP) agarose, CMC (Na- salt), alginate (sodium salt, polyguluronic and polymannuronic acid mixture), carrageenan (mixture from Irish moss) and xylan (from oat spelts). The culture was incubated at 30 °C on orbital shaker at 130 rpm for 24 h. 0.1% of the starter culture was aseptically transferred to a freshly prepared ASW medium containing single or dual or mix polysaccharides and supplemented with yeast extract under conditions mentioned above. After 48 h, the culture supernatant was obtained by centrifugation at 6360 × *g* for 15 min at 4 °C and immediately used to

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Table 1
Carbohydrase expression profile of *Microbulbifer* strain CMC-5 in presence of various polysaccharides provided as single or mix substrates. Values within the parentheses are specific activities (expressed as μmole of reducing sugar released/mg of protein per minute, [protein concentration determined by Folin Lowry method]). Unit of carbohydrase is defined as μmole of reducing sugar released $ml^{-1}\,h^{-1}$; ND – Not detected.

Polysaccharide/s	Agarase	CMCase	Alginase	Xylanase	Carrageenase
	Units and specific activities				
Agarose	1.2 (0.011)	ND	ND	ND	ND
CM Cellulose	ND	1.11 (0.014)	ND	0.266 (0.0033)	ND
Alginate	0.55 (0.006)	ND	1.12 (0.012)	0.072 (0.0006)	0.161 (0.002)
Xylan	ND	0.072 (0.0022)	ND	0.427 (0.0133)	ND
Carrageenan	0.072 (0.0022)	ND	ND	ND	0.783 (0.026)
CM cellulose + agarose	1.2 (0.025)	0.633 (0.0133)	0.189 (0.004)	0.167 (0.0033)	0.072 (0.0017)
CM cellulose + alginate	0.283 (0.016)	0.5 (0.027)	0.661 (0.036)	0.128 (0.007)	0.05 (0.003)
CM cellulose + xylan	0.383 (0.008)	0.688 (0.0144)	0.350 (0.007)	0.283 (0.006)	0.050 (0.0011)
CM cellulose + carrageenan	0.383 (0.019)	0.550 (0.027)	0.050 (0.002)	0.072 (0.0033)	0.455 (0.022)
Agarose + alginate	0.605 (0.037)	ND	0.605 (0.037)	ND	0.050 (0.0033)
Agarose + xylan	1.20 (0.007)	ND	ND	0.383 (0.023)	ND
Agarose + carrageenan	0.372 (0.011)	ND	ND	ND	0.383 (0.012)
Alginate + xylan	0.139 (0.007)	ND	0.583 (0.027)	0.361 (0.017)	ND
Alginate + carrageenan	0.139 (0.008)	ND	0.438 (0.024)	0.028 (0.002)	0.105 (0.006)
Xylan + carrageenan	0.139 (0.004)	ND	ND	0.167 (0.0044)	0.139 (0.004)
Polysaccharide mix	0.755 (0.0233)	0.405 (0.0122)	0.372 (0.012)	0.266 (0.0083)	0.527 (0.017)

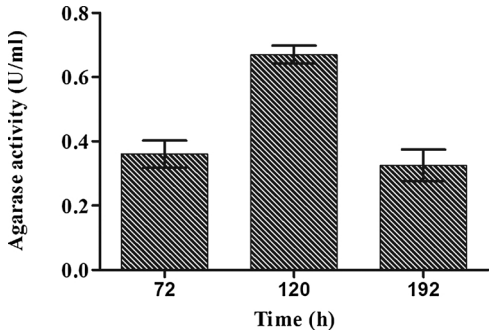

Fig. 1. Production of agarase at different time intervals by *Microbulbifer* strain CMC-5 during its growth on biomass of *Gracilaria corticata*.

assay for various carbohydrase activities.

2.2. Carbohydrase activity

0.5 ml of the culture supernatant and 2.5 ml of substrate was used for determining agarase, CMCase, alginate lyase, carrageenase and xylanase activities. 0.2% polysaccharide stock solutions of agar, alginate, carrageenan and xylan were prepared in 20 mM TrisCl (pH 7.0) whereas CMC was resuspended in 20 mM sodium citrate buffer (pH 6.5). Agarase, alginase and carrageenases activities were measured at 30 °C whereas CMCase and xylanase activities were measured at 45 °C for 90 min. Reducing sugar released was measured by dinitrosalicylic acid (DNSA) assay at 540 nm [14]. The concentration of reducing sugar released by agarase and carrageenase enzymes was obtained from galactose standard. Reducing sugars released by CMCase and alginase activities were compared with glucose standards, whereas xylanase activity was compared with xylose standard. Appropriate enzyme and substrate controls were included during the assay. The activity assays were done in triplicate and the range for standard deviation was + 0.55-1.12. One unit of carbohydrase activity was expressed as one micromole of reducing sugars released per h at respective temperatures.

2.3. Carbohydrase activity in presence of seaweed biomass

The profile of carbohydrases produced by strain CMC-5 during its growth on seaweed biomass was examined. *Gracilaria corticata* collected from the Anjuna coast, Goa, India was used as test seaweed. Axenic culture of *Gracilaria corticata* was prepared in Provasali's enriched sea water (ESP) medium according to Chen and McCracken (1993) [15]. Bacterial strain CMC-5 was grown in ASW medium containing 0.2% glucose for 24 h at 30 °C on an orbital shaker at 130 rpm. The bacterial cell pellet was obtained by centrifugation at 6360 × *g* at 4 °C and subsequently washed with sterile ASW medium. 0.1% of bacterial inoculum was added to sterile ASW medium containing 1 g of cut pieces of sterile *Gracilaria* thalli (3–4 mm) and incubated at 30 °C for 8 days on orbital shaker at 130 rpm. Samples were withdrawn at different time intervals. Agarase and CMCase activities from the culture supernatant were estimated by DNSA method as mentioned earlier. Axenic thallus of *Gracilaria corticata* aseptically cut into small pieces and incubated in sterile ASW medium without bacterial inoculum was used as control.

3. Results and discussion

In a natural system, where different ICPs form a complex array of interconnecting polysaccharides, a multiple polysaccharide degrading bacteria like *Microbulbifer* strain CMC-5 is confronted with the challenge of targeting several polysaccharides at a given time. *Microbulbifer* strain CMC-5 was isolated from decomposing seaweeds and is capable of degrading several polysaccharides [6]. Furthermore, the ability to degrade ten variable polysaccharides have been reported in *Saccharophagus degradans* 2–40, that was isolated from decaying salt marsh grass [16]. Additionally, an unknown unique bacterium degrading seven diverse polysaccharides have been isolated from *Fucus disticus* [17]. Thus decomposing salt marsh grass and seaweeds appear to be a potential niche for isolating multiple polysaccharide degrading bacteria. In addition, several strains of *Microbulbifer* with potentials to degrade multiple polysaccharides have been reported from various niches [18–21]. Although multiple polysaccharide degrading bacteria have been isolated predominantly from marine habitats, even non-marine habitats such as terrestrial rhizosphere have yielded several strains of *Paenibacillus* sp. with multiple polysaccharide degrading activities [22].

The cell wall of seaweeds contains a mixture of polysaccharides and offers a unique micro niche supporting growth of polysaccharide degrading bacteria. Although bacterial growth and induction of homologous carbohydrase in presence of single polysaccharides have been studied, inductions of carbohydrase in presence of mixed polysaccharides have not been explored. Agarose, CMC, alginate, carrageenan and xylan were used as carbon substrate either singularly or in

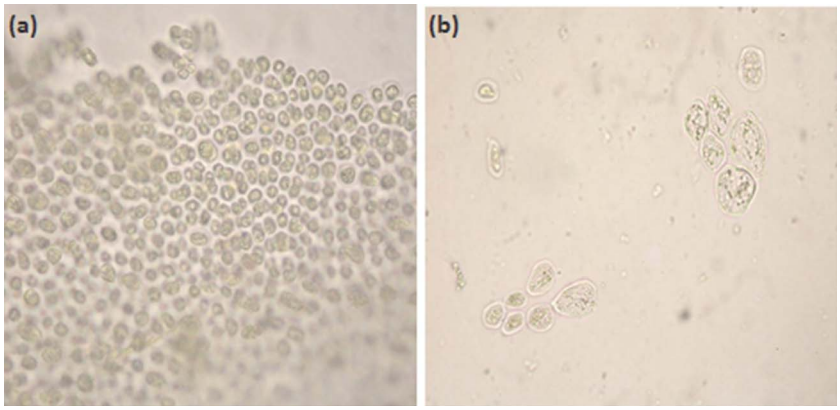

Fig. 2. *In vitro* algal thalli degradation by *Microbulbifer* strain CMC-5. (a) Control thallus of *Gracilaria corticata* as observed under compound microscope (×100); (b) Single cell detritus (×100) released from degraded thalli of *Gracilaria corticata* during growth of strain CMC-5 on seaweed biomass.

various combination of coupled substrates to study the expression of corresponding homologous or other heterologous carbohydrase in *Microbulbifer* strain CMC-5. As observed from Table 1, agarase and CMCase were best induced in presence of agar and CMC respectively. In *Microbulbifer* strain CMC-5 with the exception of agarase, no other heterologous carbohydrases were produced in presence of agarose. Similarly, in *Cytophaga diffluens* only agarase production was observed during growth on agar, whereas other heterologous carbohydrase such as CMCase and xylanase were not detected [23]. Contrary, presence of agarose as the sole carbon substrate, promoted the synthesis of agarase and other heterologous carbohydrases in *Saccharophagus degradans* 2–40 [12]. Further CMC alone induced CMCase predominantly and was accompanied by low yield of xylanase. The detection of supplementary xylanase activity in presence of CMC as a substrate could be attributed to the fact that several lignocellulolytic enzymes have been reported to depict substrate cross-specificity [24–26]. Additionally, CMC and xylan have also been reported to compete for the same active site in two enzymes purified from Onozuka-cellulase, a commercial enzyme from *T.viride* [27,28]. Moreover, cellulose has been reported to induce cellulase as well as xylanase in *Saccharophagus degradans* 2–40 [29].

Alternatively, strain CMC-5 was also grown in ASW medium supplemented with coupled polysaccharides that were incorporated in various combinations. Diversified carbohydrase activities ranging from no activity to intermediate and high activities were detected. The corresponding carbohydrases equivalent to the polysaccharide pair used in the combination for growth study were predominantly produced at higher levels in comparison to other heterologous carbohydrases (Table 1). Alginate lyase production was not observed, if alginate or CMC was the depleted polysaccharide in dual combination studies. Production of CMCase was strictly observed only when CMC was one of the substrate incorporated during dual polysaccharide combination studies. CMCase production was not observed when CMC was missing in the coupled polysaccharide studies. Additionally, only CMCase activity was detected when CMC was provided as sole substrate, although when xylan was provided as sole carbon substrate, a very low level of CMCase was detected (Table 1). Thus xylan or intermediates produced during xylan degradation might be involved in cross induction of CMCase. Furthermore, xylanase was not detected in the culture supernatant when CMC or xylan was the missing polysaccharide in dual combination studies. When microorganisms degrade different polysaccharides to respective oligosaccharides, they released small, medium or large sized oligosaccharides that enter the cell and induce the expression of enzymes responsible for respective polysaccharide degradation. Xylan degrading enzymes can be induced predominantly by medium and large sized oligosaccharides than small sized oligosaccharides [8]. Further some of the heterologous oligosaccharides might be structurally related and may cause cross induction of unrelated carbohydrases. Induction of different xylanase enzymes from *Cellulomonas cellulovorans* was observed in different substrates such as cellulose, avicel, oat spelt xylan and birchwood xylan and is presumably due to intermediates which may cross induce other pathways [30].

Thus when single homologous polysaccharides (agar, alginate, CMC, carrageenan or xylan) was provided as one of the growth substrate, none were successful in inducing production of all the five carbohydrase in strain CMC-5. Further all the five carbohydrases were produced only when CMC was included as one of the polysaccharide during coupled polysaccharide studies (Table 1). Productions of all five carbohydrases were not observed in other coupled polysaccharide studies. However, when a blend of all five polysaccharides was provided as the substrate for growth, all the five carbohydrases were produced although the activities detected were comparatively lower than those observed with respect to control (Table 1). Thus strain CMC-5 is capable of attacking all the test five polysaccharides when they are a part of growth substrates. To our knowledge, this is the first report where simultaneously all five carbohydrases were produced in presence of dual polysaccharide substrates such as agar and cellulose.

Although, growth of terrestrial fungi on marine seaweed biomass has been previously reported [31], recently growth of bacterial strain CMC-5 using marine seaweed biomass has also been described [6]. Nikolaeva et al. (1999) reported the production of agarase, CMCase and protease during growth of various fungi on seaweed biomass. However, the level of agarase activities produced by various fungi after five passages on medium containing *Palmaria palmata* were lower when compared to agarolytic bacterium infusorium consortium used during the experiment [23]. Although strain CMC-5 is a multiple polysaccharide degrader, the growth of bacterial strain CMC-5 on biomass of *Gracilaria corticata* elicited essentially the production of agarase enzyme with maximum activity peaking at 120 h (Fig. 1). CMCase, alginate lyase and carrageenase activities were not detected in the culture supernatant. The cell wall of *Gracilaria* is composed primarily of agar and cellulose with traces of carrageenan. The detection of only agarase during initial stages of seaweed biomass degradation study suggests that the strain is capable of degrading agar for deriving energy for growth as described previously [6]. Since cellulose is intermeshed in the agar backbone, cellulose is not easily accessible for strain CMC-5 leading to failure to induce CMCase activity in the present study. Also since degradation of seaweed cell wall may be sequential, agarase activity may be induced during the initial stages followed by CMCase induction at later stages. Further, the growth of strain CMC-5 on marine seaweed biomass was accompanied by release of cell clumps as well as few single cells as observed under compound microscope which is an indication of degradation of interstitial matrices within the seaweed (Fig. 2). Thus the carbohydrase produced by strain CMC-5 can be engaged in production of single cell detritus from seaweeds which can be used as a potential feed in aquaculture industry. The concurrent productions of all five carbohydrases also suggest that this strain can be exploited in bioremediation of algal wastes, especially if the medium is seeded with dual mixture of polysaccharides with CM cellulose as one

of the substrate.

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