Available online at: www.mbai.org.in doi:10.6024/jmbai.2020.62.2.2183-14

Enzyme profiling of macroalgal endophytes: An attempt to uncover the arsenal of novel biocatalysts

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Received: 09 April 2020 Accepted: 30 Dec 2020 Published: 10 Jan 2021

Original Article

Abstract

Endophytes, an integral part of the plant microbiome, are mainly fungi that reside within intercellular tissues of plants without triggering any disease symptoms. Like other microbial invasions, endosymbionts produce extracellular hydrolases as a resistance mechanism to overpower the immune responses of the host or to utilize host nutrients for survival. About 133 endophytes isolated from eleven macroalgae of Kerala Coast, India were screened for their catalytic potential. Screening was done for a total of ten enzymes by plate assay method. Out of the 133 isolates screened, 123 isolates expressed asparaginase activity (92.5 %). More than 60% of isolates exhibited amylase (89.5 %), lipase (88 %), gelatinase (78.9 %) and glutaminase (69.2 %) activity. Quantitative estimation of asparaginase production in Czapek Dox medium by potent strains revealed that *Penicillium chrysogenum* exhibited maximum asparaginase production. The glutaminase free asaraginase production of *P. chrysogenum* would be a potential source to explore for therapeutic applications.

Keywords*: Endophyte, Fungi, Macroalgae, Hydrolytic Enzyme, Asparaginase*

Introduction

For the past few decades, fungi rule the enzyme industry by supplying 60% of currently used industrial enzymes (Ostergaard and Olsen, 2010). Relating the absorptive mode of nutrition and ecological role in decomposition cycle, a vast variety of hydrolytic enzymes which can be used in biotechnological processes such as lyase, cellulase, glucosidase, inulinase, ligninase, amylase, keratinase, protease, nuclease, chitinase, phytase, xylanase and lipase can be expected from fungi (Bonugli-santos *et al*., 2015; Suryanarayanan, *et al.,* 2012). Even though many reports are available on the enzyme production potential of terrestrial mycota, works on marine fungi remain scarce. A potential source is the barely exposed marine fungi i.e., those which dwell the internal tissues of mangrove, algae, sea grass, sponges and tunicates in symbiotic harmony, '*the marine endophytes'* (Raghukumar, 2008). Since occupying a highly hostile environment, they might possess novel enzyme systems which are essential for effective host tissue colonization (Bhagobaty and Joshi, 2012). Like other microbial invasion, endosymbionts generally produce extracellular hydrolases as a resistance mechanism to overpower the immune response of host or to utilize host nutrients for survival (Tan and Zou, 2001). Hence, most of the endophytes are reliable source of cell wall degrading enzymes

like pectinase, laccase, xylanase, chitinase, cellulase and lipase (Moy *et al*., 2002, Li *et al*., 2004, Promputtha *et al*., 2005). These enzymes have profuse applications that can be employed to address the problems in bioremediation, food security, energy production and therapeutics. Anticancer properties of fungal asparaginase and glutaminase are also worth to discuss, as the industry primarily depends on bacterial asparaginase for cancer therapy. Recently, scientists are forced to find an alternative because of certain hypersensitivity reactions of bacterial asparaginase (Suryanarayanan *et al.,* 2012).

It is important to consider that the secondary metabolites synthesized by a fungus may rely on its ecological niche and the persistent metabolic interactions between guest and host may enhance the synthesis (Tenguria *et al.,* 2011). In order to thrive in a highly hostile environment (prolonged periods of sunlight exposure, sharp variation in moisture and salt concentration, changing tides, abundant microorganisms and herbivore insects), macroalgae and their endosymbionts might possess a battery of potent biocatalysts (Schulz *et al.,* 2002). Moreover, macroalgae hold the heritage of extensive therapeutic possibilities that have been widely exploited in ancient era. Most of the algal species of marine realm have not been well assessed for endophytic assemblages and its catalytic properties even though they represent the second largest source of marine fungi (Jones, 2000). The present study was focussed on the hydrolytic enzyme production potential of macroalgae associated endophytic fungi with special reference to asparaginase.

Material and methods

Source of endophytic fungi

Endophytic fungi (133 nos.) isolated from 11 macroalgae *viz., Sargassum wightii, Caulerpa peltata, Ulva fasciata, Dictyota dichotoma*, *Halymenia venusta* collected from Kovalam coast, Trivandrum, Kerala (8.389915° N; 76.975173° E) and *Hypnea musiformis, Grateloupia lithophila, Enteromorpha compressa, Ceramium diaphanum, Padina tetrastromatica* and *Chaetomorpha antennina* from Palakkulam Beach, Calicut, Kerala (11.462134° N; 75.663587° E) were used for the present study. Purity of the isolates was confirmed by culturing on malt extract agar plates.

Screening for hydrolytic enzyme production

All the endophytes were screened for the production of various extracellular hydrolytic enzymes, *viz.*, amylase, protease, cellulase, lipase, laccase, tyrosinase, ligninase , asparaginase, glutaminase and chitinase. Screening was carried out in malt extract agar medium supplemented with corresponding substrates followed by incubation for 3- 4 days at 28 ± 2 °C (Table 1). Modified Czapek Dox medium (Dextrose, 0.2%; K_2HPO_4, 0.152%; $MgSO_4.7H_2O$, 0.052%; KCl, 0.052%; $CuNO_3.3H_2O$, 0.003%; $ZnSO_4.7H_2O$, 0.005%; $FeSO_4.7H_2O$, 0.003% and pH 6.2) in seawater supplemented with L-asparagine/ L-glutamine (1.0 %) was used for the screening of asparaginase/ glutaminase production. A pH indicator, Phenol red (2.5%) prepared in ethanol was added to the Czapek Dox medium (300μl/L). Endophytes were inoculated and incubated for 3 days at 28 ± 2 °C. Formation of a pink zone around the colony was noted and recorded (Gulati *et al*., 1997).

Table 1. List of substrates and reagents used for enzyme assays

Sl. No.	Enzyme	Substrate	Reagents used for detection	Observation
1	Amylase	Starch-1%	Lugol's iodine	Clear zone
2	Gelatinase	Gelatin -1%	Mercuric chloride	Clear Zone
3	Lipase	Tributyrin-1%	-	Clear zone
4	Cellulase	Carboxy Methyl Cellulose- 1%	Congo red	Clear zone
5	Chitinase	Colloidal chitin-5%	-	Clear zone
6	Tyrosinase	Tyrosine-0.5%	-	Clear zone
7	Ligninase	Tannic acid-0.5% or Methylene blue-0.02%	-	Clear zone Clear zone
8	Asparaginase	Asparagine-1%	Phenol red	Pink coloration
9	Glutaminase	Glutamine-1%	Phenol red	Pink coloration
10	Laccase	Naphthol-0.005%	-	Purple zone

Quantitative estimation of crude L-asparaginase

Based on plate assay, 12 isolates were selected for further study. Submerged fermentation was employed for the production of crude L-asparaginase. Fungal cultures were inoculated into Czapek Dox broth and incubated at 37 ± 2 °C for 6 days, followed by filtration through Whatman No.2 filter paper in order to obtain cell free filtrate. This filtrate was used as crude enzyme to estimate asparaginase activity.

A reaction mixture containing 0.1 mL of 40m M L- asparagine, 0.5 mL of 0.5M Tris- HCl buffer (pH 8.2), 1.0 mL of appropriately diluted crude enzyme (cell free filtrate of fungal culture) and 0.4 mL of distilled water was incubated at 28 ± 1 °C for 30 minutes. The reaction was stopped by adding 0.5 mL of 1.5M tricholoroacetic acid (TCA). Subsequently added 0.2 mL of Nessler's reagent and 3.7 mL of distilled water to 0.1 mL of above reaction mixture and incubated for 20 minutes. Hydrolysis of L-asparagine was determined by calculating the ammonia released during the reaction by measuring the absorbance at 450 nm. One international unit (IU) of L-asparaginase is the

amount of enzyme needed to liberate 1 μmol of ammonia in 1 minute at 28 $\pm$ 1 °C (Imada *et al.*, 1973).

Results and discussion

All the algicolous endophytes were screened for extracellular enzyme productions *via* agar plate assay (Fig. 1). Out of the 133 endophytic fungi screened, more than 50% of isolates exhibited amylase, lipase and gelatinase activity (Fig. 2). Even though there are reports on amylolytic fungal endophytes, very few positive strains have been reported (Stamford *et al.*, 2002; Maria *et al.*, 2005). In our investigation, around 119 isolates (89.5%) exhibited amylase production, recommending endophytes as a reliable source of amylase. Though the use of amylase in starch based industries has been prevalent for decades, only a few selected strains of fungi and bacteria meet the criteria for commercial production (Souza and Magalhães, 2010). Likewise, microbial gelatinase and lipase are extensively used in feed, baking and detergent industry (Sharma *et al.*, 2001; Gupta *et al.*, 2002). In this context, it is worth to consider the gelatinase (79%) and lipase (88%) producing endophytes from marine source. Similar scenario has been observed in mangrove endophytes as well as salt marsh fungi (Gessner, 1980; Job *et al.*, 2015).

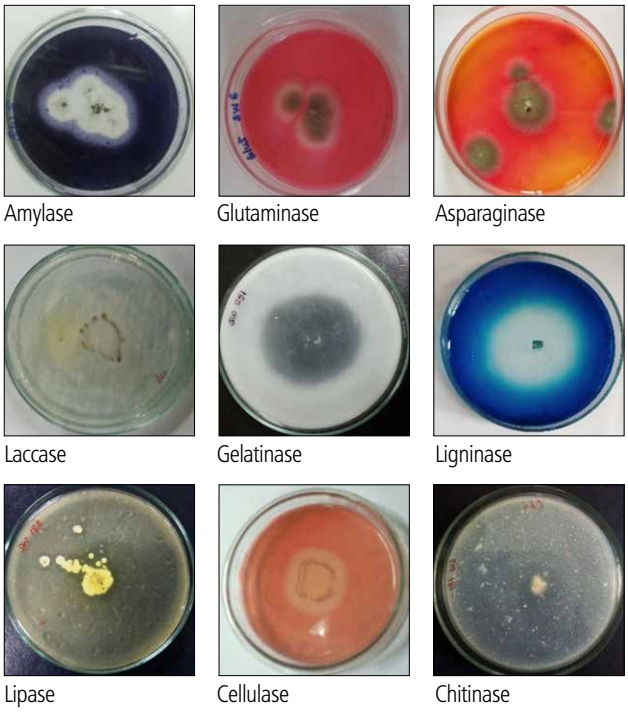

Fig. 1. Hydrolytic enzyme production by macroalgae associated endophytic fungi on respective agar media

Being a part of decomposition cycle, marine endophytes are expected to produce an array of enzymes like cellulase, chitinase, laccase and ligninase essential to degrade organic matter (Raghukumar *et al.,* 1994 a; Sharma *et al.*, 1994; Vázquez de Aldana *et al.,* 2013). Generally, the industry depends on soil-born fungi for cellulase production (Pandey *et al.,* 2000). Variety of fungi isolated from marine algae as well as mangroves were found to be active producers of cellulase (Raghukumar *et al.*, 1994a, 2004). In the present study, 37.6% of the endophytes were cellulolytic. Likewise, efforts have been made to study the salt induced chitinase globally (Hong and Hwang, 2002; Arfi *et al.,* 2013). Moreover, potential therapeutic applications of chitin derivatives have aroused a new interest in chitin-modifying enzymes (Suryanarayanan *et al.*, 2012).

Herein, we could come across with 14% chitin degrading endophytes with *Aspergillus nomius* (SW351) expressing maximum chitinase production comparable with previous reports (Narayanan *et al.,* 2013; Venkatachalam *et al.*, 2015). Among all, only *A. chevalieri* (SW371), inhabitant of *Padina tetrastromatica,* displayed the production of both chitinase and laccase. The ligninase and laccase potential of marine fungi from mangroves and sea grasses have already been reported (Raghukumar *et al.,* 1994b; Kumaresan and Suryanarayanan, 2002; Martinho *et al.*, 2019). Ligninase and laccase activity were noticed in the current investigation also. Since the effluents of paper pulp and dyeing industries have high

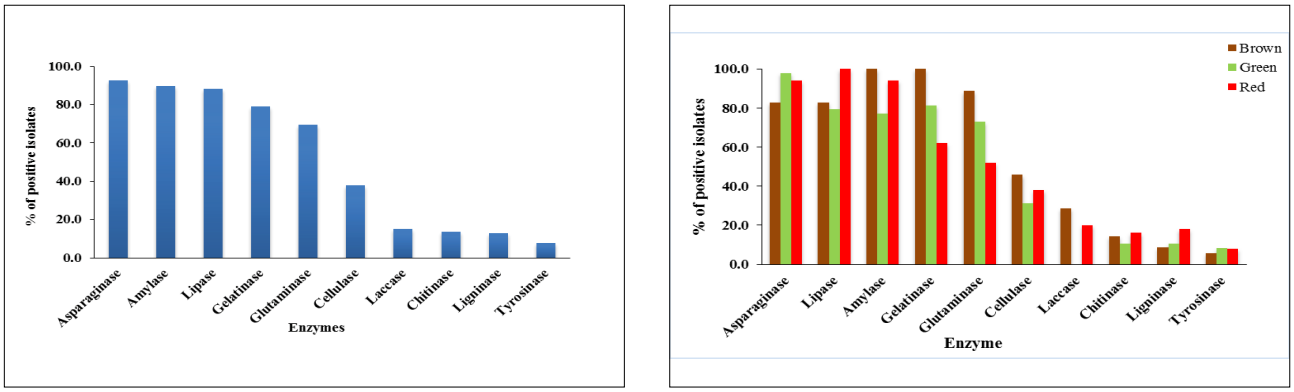

Fig. 2. Hydrolytic enzyme profile of endophytic fungi isolated from macroalgae of Kerala coast: (A) Total endophytes (B) Algae based

salt content, salt tolerant enzymes may be appropriate for the bioremediation. Hence, among the various hydrolytic enzymes, the ligninolytic system is of great significance in environmental remediation.

Application of tyrosinase is mainly concentrated in the detoxification of water and soil systems contaminated with phenolic compounds (Martorell *et al*., 2012) as well as in pharmaceutical industry (Seetharam and Saville, 2002). Very few reports are available on endophytic tyrosinase (Zaidi *et al.,* 2013) and hence, the tyrosine utilizing endophytes (7.5 %) are worth to consider.

Screening of pharmaceutically important enzymes, L-asparaginase and L-glutaminase indicated that 123 isolates (92.5 %) expressed asparaginase activity, whereas 92 strains were positive for glutaminase (69 %) production, especially endophytes of genera *Aspergillus* and *Penicillium*. The algae wise analysis also showed that maximum number of endophytes were potent producers of these two therapeutic enzymes. Explorations on enzymes such as asparaginase and glutaminase have recently been intensified due to their valuable anticancer activity against acute lymphoblastic leukemia (Gallagher *et al.,* 1999; Hatamzadeh *et al*., 2020). However, clinically available asparaginase is of bacterial origin, which elicits immune responses leading to allergic reactions (Goodsell, 2005; Schrey *et al.,* 2010). Asparaginase from a eukaryote like fungi could be an effective solution to this problem (Thirunavukkarasu *et al.,* 2011). Present study uncovered 93 % of positive strains for asparaginase, unlike Thangavel *et al* (2013) who obtained only 60 % positive strains from macroalgae. Additionally, the quantitative estimation of selected strains confirmed maximum asparaginase production in *Penicillium chrysogenum*. Further, 69.2 % of the tested endophytes showed glutaminase production, but a sharp variation from this finding was reported by Sajitha *et al* (2013). Their report reviewed only 20 % glutaminase producers from endophytes of different seaweeds with maximum production by *Penicillium* sp. Though there are many reports on the potential of marine microbes in glutaminase production (Renu and Chandrasekharan, 1992; Iyer and Singhal, 2009; Siddalingeshwara *et al*., 2010), information on asparaginase production remain very less (Usha *et al*., 2011; Thirunavukkarasu *et al*., 2011). Even though both are anticancer enzymes, some studies have reported side effects of L-asparaginase caused by allergic responses and anaphylaxis due to the associated glutaminase activity (Muller and Boos, 1998). Hence, based on plate assay, we selected ten potent glutaminase free asparaginase producers for quantitative estimation (Fig. 3). L-asparaginase production in the culture broth ranged from 109.8 to 443.5 IU/ml (Table 2). Maximum production was exhibited by SW4 (*P. chrysogenum*) followed by SW121 (*P. chrysogenum*) and SW32 (*A. sydowii*).

The present study is focused on the catalytic property of marine algicolous endophytes. Production of therapeutic enzymes *viz.*,

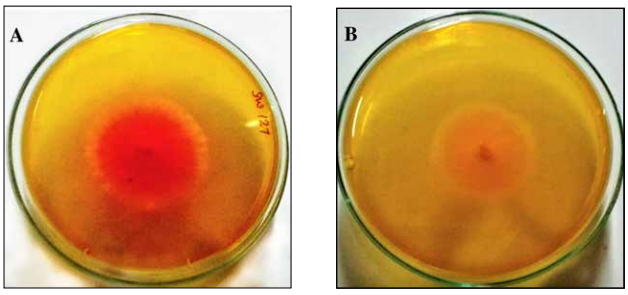

Fig. 3. Plate assay showing the glutaminase free asparaginase activity of an endophytic fungus: (A) Asparaginase positive (B) Glutaminase negative

Table 2. Quantitative estimation of asparaginase production in endophytes

Host macroalgae	Endophytic fungi	Asparaginase in crude extract (IU/ml)
Sargassum wightii	*Aspergills flavus* SW 1	145.22
Sargassum wightii	*Penicillium chrysogenum* SW 4	443.47
Ulva fasciata	A*spergillus sydowii* SW 32	332.72
Halymenia venusta	*Penicillium chrysogenum* SW 121	335.94
Halymenia venusta	*Penicillium rubens* SW 130	307.90
Hypnea musiformis	*Aspergillus sydowii* SW 152	317.10
Hypnea musiformis	*Penicillium citrinum* SW 171	109.83
Ceramium diaphanum	*Xylaria feejeensis* SW 181	314.34
Dictyota dichotoma	*Daldinia eschscholtzii* SW 211	326.29
Chaetomorpha antennina	*Fusarium equiseti* SW 302	294.12

asparaginase and glutaminase by 70% of the isolates were noteworthy besides the significant percentage of amylase, lipase and gelatinase positive forms among the endophytic fungi. Cellulase, tyrosinase, ligninase, laccase and chitinase production were observed only in limited number of isolates. Percentage of hydrolytic enzyme producing forms was more among the isolates from brown and red algae compared to green algae. Study reveals the potential of endophytes as a prominent source of hydrolytic enzymes for industrial applications.

Acknowledgements

The first author is thankful to the Department of Science and Technology (DST), Government of India for the award of INSPIRE Fellowship. We thank Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, India for providing necessary facilities.

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