BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, October 2014. Vol. **11**(Spl. Edn. 1), p. 307-312

Endophytic Fungal Cellulase for Extraction of Carrageenan and its Use in Antibiotics Amended Film Preparation

Radhakrishnan Muthezhilan[1]*, Kuzhandaivel Jayaprakash[1], Ramachandran Karthik[1] and Ajmath Jaffar Hussain[2]

[1]Department of Marine Biotechnology, AMET University (U/S of UGC Act 1956)
Kanathur, Chennai 603112, India
[2]Centre for Marine Bioprospecting, AMET University (U/S of UGC Act 1956)
Kanathur, Chennai 603112, India.

doi: http://dx.doi.org/10.13005/bbra/1424

(Received: 15 August 2014; accepted: 10 October 2014)

The use of non biodegradable plastic based packaging materials has been stopped in several food industries due to their disadvantages, so bio based polymers like polysaccharides are the alternative source for the preparation of films in food packaging. Thus, the present work was aimed to prepare the carrageenan (polysaccharides) based antibiotics amended films to prevent the food borne microbial pathogen in packed foods to extend its shelf life. In this study, morphologically 25 different endophytic fungal strains were isolated from different salt marsh plants, such as, *Sweda monica* and *S.maritima* were collected from Kelambakam Coastal area, East Coast of Tamil Nadu, India and they were named as, AEF51 to AEF75. All the fungal isolates were screened for cellulase activity and the maximum cellulase producing potential strain AEF58 was mass cultured. After the partial purification of cellulase it was lyophilized and stored for further use. The red seaweed *Amphiroa anceps* was collected from *Kootapuli Village*, Valliyoor Taluk, Tirunelveli District, Tamil Nadu, India. From that, the carrageenan was extracted by biological method using endophytic fungal cellulase. After the extraction of carrageenan the antibiotics such *as Amoxycillin, Tetracycline, Chloroamphenical, Erythromycin, Doxycyoline, Ofloxacin, Cephalexin, Dictoxacilin* and *Ampicillin* were amended and different films were prepared using standard protocols. All the films were tested against different seafood borne pathogens such as, *E.coli, Vibrio cholerae, V.parahaemolyticus, Salmonella* sp, *Shigella* sp and *Listeria* sp (Obtained from AMET Microbial Culture Collection Centre). Among them, the *Cephalexin* amended film was showed the maximum zone of inhibition (ZOI) against all the tested pathogens. From the results, the study concluded that, the extraction of carragenan by biological substances increase the production and antibiotic amended carrageenan based films will be used for food packaging to control the pathogenic microbes and preserve the foods from spoilage.

Key words: Seaweeds, Carrageenan, Antibacterial Activity, Seafood Packaging.

In general, food can be associated with many potential risks, especially to microbiological contamination due to many factors including packaging and storage method . So, the quality of food is most important because of the increasing demands in markets. Nowadays, the major goal of the food processing industries is to provide safe, wholesome and acceptable food to the consumer and control of microorganisms (Karthik *et al*., 2013). The use of non biodegradable plastic based packaging materials are being stopped in several food industries due to their palatability and biodegradability, So, there is an urgent need of bio based polymers as an alternative source for food packaging (Abdou and Sorour, 2014). Recently, there are some considerable research has been conducted to develop bio based polymers made

* To whom all correspondence should be addressed.
E-mail: mycomuthu@gmail.com

from a variety of agricultural or food waste products. There are some biopolymers include starches, cellulose derivatives, chitosan or chitin, proteins (animal or plant-based) and lipids, to make thin films and coatings to cover fresh or processed foods to extend their shelf life. Normally, the use of bio based films have extra advantages such as edibility, biocompatibility, esthetic appearance, barrier to gasses properties, non-toxicity, non-polluting and in addition it can also act as carriers of foods additives (antioxidants, antimicrobials) and have been particularly considered in food preservation (Sallam, 2007).

The marine macro algae, seaweeds are able to produce different types of sulfated polysaccharides (carrageenans) linear polysaccharides that occur in the cell wall and intercellular matrix of seaweeds (Falshaw *et al.*, 2005). The primary structure of the carrageenans are based on an alternating disaccharide repeating sequence of ß-D-galactose (linked at position 3) and ±-Dgalactose (linked at position 4), which have extensive applications (such as thickeners, stabilizers, gelling agents, cosmetics, toiletries and toothpaste) in food and pharmaceutical Industries (Abdou and Sorour, 2014). Also, recently, it is reported that, incorporating microbialcidal or microbialstatic agents into such a packaging system or by using active antimicrobial polymeric materials into food packaging material can extend the lag period and reduce the growth rate of pathogenic and non pathogenic microorganisms and also extend the shelf stability and promote safety (Assefa and Admassu, 2013).

Thus, the present study was aimed to extract the carrageenan (polysaccharides) using endophytic fungal cellulase as substrate to prepare different antibiotics amended films to prevent the food borne pathogens in packed foods to extend its shelf life.

MATERIALS AND METHODS

Isolation of Endophytic Fungi

For isolation of endophytic fungi from different salt marsh plants, such as, *Sweda monica* and *S.maritima* were collected from Kelambakkam Coastal area, East Coast of Tamil Nadu, India. The plant materials were washed under running tap water for 10 minutes to remove the dirt. Before surface sterilization, the materials such as, leaf, stem and root were cut into small pieces (0.5 cm) and they were sterilized with 70% ethanol and 1.0 % sodium hypochlorite (NaOCl) (v/v) for 1 minute and further cleaned by passing through sterile distilled water (Jeffrey *et al*., 2008). The surface sterilized samples were placed on petri plate containing potato dextrose agar (PDA) with 200 mg/L concentration of streptomycin to suppress the bacterial contamination. The parafilm wrapped petriplates were incubated for 7 days at room temperature. After the incubation period, morphologically different pure fungal cultures were sub cultured in potato dextrose agar (PDA) slants for further study (Subbulakshmi *et al*., 2012).

Screening of cellulase producing endophytic fungi

All the isolated morphologically different endophytic fungal strains were (individually) inoculated in 50ml of potato dextrose broth (PDB) and kept for 36 hrs incubation at 30ºC. After the incubation period, all the tubes were centrifuged at 3000 rpm for 20 minutes. 100µl of obtained each culture filtrates were added in the wells on selective carboxymetyl cellulose agar medium (containing (1%): NaNO3 2.0, KH2 PO4 1.0, MgSO4 .7H2O 0.5, KCl 0.5, carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 20.0) and incubated at 30ºC for 3 days. After the incubation period all the plates were flooded with 1% Congo red solution for 15 minutes then de-stained with 1M NaCl solution for 15 minutes. Based on the zone of decolorization around the well the potential endophytic fungal strain were selected and stored in PDA plates for further use (Charitha Devi and Sunil Kumar, 2012).

Identification of cellulase producing potential endophytic fungal strain

The potential endophytic fungal strain was identified by referring standard mycological books and manuals (Gillman, 1959; Gillman; 1998; Subramanian, 1971; Ellis, 1971; Ellis, 1979).

Mass culture of cellulase producing endophytic fungal strain

The inoculums (10%) of cellulase producing potential endophytic fungal strain from potato dextrose broth (PDB) was transferred to 1L flask containing 500ml production media and, incubated at 150 rpm, 30p C for 5 days (Acharya *et al*., 2008).

Extraction cellulase from endophytic fungal strain

After the incubation period, the culture was filtered using Whatman No. 1 filter paper and the filtrate was further centrifuged at 3000 rpm for 20 mins. The insoluble materials and cell debris were separated. Further the culture filtrate was treated by ammonium sulphate precipitation and dialysis. The activity of partially purified cellulase was determined by according to the method described by Ghose (1987). Then, the partially purified cellulase was lyophilized and pre-freezed and stored for further use (Sathyavrathan and Krithika, 2013).

Collection of Seaweed

The red seaweed *Amphiroa anceps* was collected from *Kootapuli* Village, Valliyoor Taluk, Tirunelveli District, Tamil Nadu, India. After the sampling, the seaweed was immediately washed several times with clean water in order to remove non-algal materials and it was sun dried.

Extraction of carrageenan

20g of grinded seaweed was mixed with 200ml distilled water and 0.2g of cellulase was added into the mixture and boiled in water bath with shaker at 50^0C for 1 hour. After that, the suspensions were obtained by centrifuging at 5000 rpm at 4^oC for 15 minutes (Fraction 1). Then, one volume of supernatant was poured into two volumes of 2-propanol and the liquors were removed by centrifuging at 12000 rpm (4^oC) for 30 minutes (Fraction 2). The fractions were dried under rotary evaporator and recovered in a freeze-dried state and the weight of the dried caraageenan sample is recorded. Freeze dried sample was grinded into powdered form and stored in a sealed bottle for further analysis (Soovendran *et al*., 2009). The percentage of carrageenan yield was obtained using the following formula:

Carrageenan Percent (%) = Dry weight carrageenan (g) X 100% / Weight of the extracted sample (g)

Preparation of Carrageenan based antibiotics amended films

Carrageenan based antibiotics amended films were prepared with slight modifications by the method described by Eraricar *et al*., 2009. The obtained carrageenan (1% w/w) was dissolved in 10ml of distilled water and it was kept in water bath at 80°C for 10 minutes. Then 0.75% of plasticizer (50% PEG and 50% glycerol) and 0.1 gm of each antimicrobial agents such as, *Amoxycillin, Tetracycline, Chloroamphenical, Erythromycin, Doxycyoline, Ofloxacin, Cephalexin, Dictoxacilin* and *Ampicillin* were added in separate carrageenan solution (The film without antibiotic was treated as control) and the mixture was transferred into petri dishes (100 mm diameter and 15 mm deep) and spreaded with a 2mm spacer and placed in a hot air oven at 95°C for 24 hours. After that, upon cooling each film were peeled from the plate and placed in a polyethylene bag and stored in a constant temperature and humidity chamber (25^0C, 50% RH) for 3–4 days before testing (Zinash and Shimelis, 2013).

Determination of antimicrobial effect of films against different seafood borne pathogens

All the prepared different carrageenan based antibiotics amended films were cut in a small pieces (6mm diameter discs) to determine the antibacterial effect against different food borne pathogenic bacteria such as, *E.coli, Vibrio cholerae, V.parahaemolyticus, Salmonella* sp, *Shigella* sp and *Listeria* sp by the disc diffusion assay method. All the pathogens (0.2ml) were respectively swabbed on Mueller Hinton agar plates and the film discs were placed and incubated at 37°C for 24 hrs. After the incubation period the zone of inhibition (ZOI) around the discs was recorded (Zinash and Shimelis, 2013).

RESULTS AND DISCUSSION

Generally, food packaging materials or films are prepared by using several synthetic antimicrobial chemicals such as, organic or inorganic acids, metals, alcohols, ammonium compounds or amines have been incorporated due to their antimicrobial property in films against food borne pathogens. Nowadays, due to the increasing consumer health concern and the growing demand for healthy foods have stimulated the use of natural bio based enzymes incorporated polysaccharides or starch based films for food packaging (Vicentini, 2003). Carrageenans are a family of sulfated, linear polysaccharides that occur in the cell wall and intercellular matrix of numerous species of red seaweeds (Abdou and Sorour, 2014). In this present study, two different salt marsh plants such as, *Sweda monica* and *S.maritima* were collected from Kelambakkam Coastal area, East Coast of Tamil Nadu, India. Totally 25 different endophytic fungal strains were isolated from different segments (Leaf,

Stem, and Root) of salt marsh plants, and they were named as AEF51 to AEF75. Among that, 13 fungal strains were isolated from *S.maritima* and 12 fungal strains from *Sweda monica* respectively. When comparing with plant segments, most of the fungal strains were isolated from root, stem and leaf respectively (Table 1).

Table 1. Isolation of Morphologically different endophytic fungi from salt marsh plants

Plant Segments	Name of the salt marsh plants	
	Sweda monica	*S.maritima*
Leaf	3	4
Stem	4	2
Root	5	7
Total No. of strains	12	13

While screening all the isolated 25 endophytic fungal strains by cellulase activity, the strain namely AEF58, AEF61 and AEF69 have showed positive results among that, the strain AEF58 have showed maximum (16mm) zone of inhibition (ZOI) around the wells in carboxymetyl cellulose agar medium plates (Fig 1). Based on the cultural, morphological, microscopic characterstics, it is identified that the potential cellulose producing endophytic fungal strain AEF58 was belonging to *Aspergillus* sp. Similarly, Sri Lakshmi and Narasimha, 2012, also observed, cellulose production in carboxymetyl cellulose agar medium plates by *Aspergillus* sp. Sadaf Jahangeer *et al*., 2005, have reported that, the majority of *Aspergillus* sp can able produce cellulase compared to other fungal strains. In general, the cellulase enzyme produced by *Aspergillus* sp can able to breakdown the seaweed cell wall, so it can be used as for carrageenan extraction, to obtain it from the cell wall of seaweed as well as to obtain higher carrageenan yield (Soovendran *et al*., 2009). Hence, the potential cellulase producing endophytic fungi *Aspergillus* sp (AEF58) mass cultured for cellulase production and the obtained cellulase was lyophilized and stored for further use.

In general, the higher yield of carrageenan from seaweed depends upon the handling and input technologies (Mochtar *et al.,* 2013). Whereas extracting the carrageenan from the seaweed *Amphiroa anceps* using endophytic fungal cellulase by biological extraction, 52% of yield was observed. These results were comparatively better than Soovendran *et al*., 2009. In their studies, they observed, the highest yield during cellulase-treated extraction (45%) followed by traditional boiling (37.5%) and the lowest were observed with fungal treatment (37%) when extracting the carrageenan from the red seaweed *Amphiroa anceps* using cellulase as substrate. Mishra *et al*., 2006, also reported that, the pretreatments of dried seaweed with NaOH, KOH and Ca (OH) 2 followed by pressure-cooking showed relatively higher yield of carrageenan, but the viscosity, clarity and the texture of the gel were found to be poor. However, the gel strength is the main physical properties of carrageenan because it shows the ability of carrageenan gel strength formation (Glicksman, 1983).

The development of natural antimicrobial agents for preparing biopolymers based films for food packaging and its commercialization will be a great step towards attaining sustainability in food

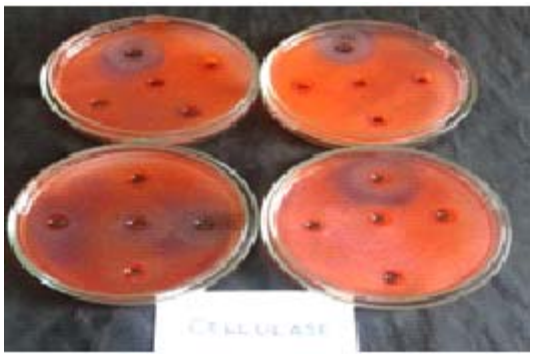

Fig. 1. Cellulase activity in CMC agar plates

Fig. 2. Antibacterial activity of carrageenan based antibiotics amended films against different food borne pathogens

packaging applications (Weber *et al*., 2002). So, in this study, carrageenan based different antibiotics (such as *Amoxycillin, Tetracycline, Chloroamphenical, Erythromycin, Doxycyoline, Ofloxacin, Cephalexin, Dictoxacilin* and *Ampicillin*) amended films were prepared. Whereas checking the antibacterial effect against different seafood borne pathogens such as, *E.coli, Vibrio cholerae, V.parahaemolyticus, Salmonella* sp, *Shigella* sp and *Listeria* sp, among them, the film prepared by using *Cephalexin* has showed the maximum zone of inhibition (ZOI) against all the tested pathogens (Fig 2 & 3). Zinash and Shimelis, 2013, also reported that, the starch-based films prepared from 90-100% (w/w) starch and 0-10% (w/w) bioactive component (saponin) have showed maximum inhibition *Escherichia coli, Salmonella typhi and Entrobacter erogenous*. Sunil, 2012 have reported that, the higher inhibitory activity shown by the antimicrobial films could make them more reactive against bacterial cells.

In general, the films prepared by using renewable sources contain various components (flavorings, colorings, sweeteners) and also they are anticipated to degrade more readily than polymeric materials. From the results, the work also suggested to use this carragenan based antibiotic amended films in food industries for food packaging to control the pathogenic microbes and preserve the foods from spoilage.

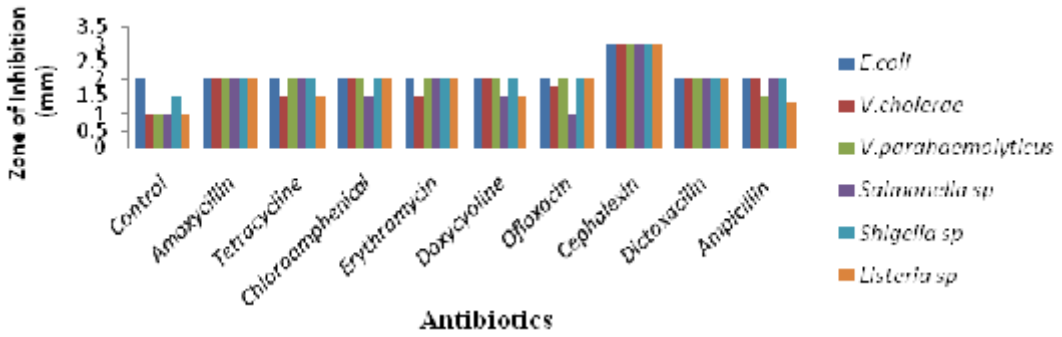

Fig. 3. Antibacterial activity of antibiotic amended films against seafood borne pathogens

REFERENCES

1. Abdou, E. S. and Sorour, M. A., Preparation and characterization of starch/carrageenan edible films. *International Food Research Journal* 2014; **21:** 189-193.
2. Acharya, S. N.; Many, A. M.; Schroeder, A.; Kennedy, P.; Savytskyy, F. M.; Grubb, O. P.; Vincent, J. T.; Friedle, J. A.; Celerin, E. A. and Maillet, M.D.S., *Coprinus cinereus* rad50 mutants reveal an essential structural role for Rad50 in axial element and synaptonemal complex formation, homolog pairing and meiotic recombination. *Genetics* 2008; **180:** 1889-1907.
3. Assefa, Z. and Admassu, S., Development and Characterization of Antimicrobial Packaging Films. *Journal of Food Processing Technology* 2013; **4:** 235.
4. Charitha Devi, M. and Sunil Kumar, M., Production, Optimization and partial purification of cellulose by *Aspergillus niger* fermented with paper and timber sawmill industrial wastes. *Journal of Microbiology and Biotechnology Research* 2012; **2:** 120- 128.
5. Ellis M.B., Dematiaceous Hyphomycetes. Common Wealth Mycological institute, England., 1971, 312.
6. Ellis M.B., Dematiaceous Hyphomycetes. Common Wealth Mycological institute, England., 1979, 304.
7. Elsabee, M. Z. and Abdou, E. S., Chitosan Based Edible Films and Coatings: A Review. *Materials Science and Engineering* 2013; **33:**1819–1841
8. Eraricar, S.; Ida IM. and Nozieana, K., Structural Characterization and Physical Properties of Antimicrobial (AM) Starch-Based Films. *World Academy Science Engineering Technol*ogy 2009; **55:** 432-440.
9. Falshaw, R.; Furneaux, R. H. and Stevenson, D. E., Structural analysis of carrageenans from the red seaweed *Callophyllis hombroniana* Mont. Kutz (Kallymeniaceae, Rhodophyta). *Carbohydrate Research* 2005; **340:**1149–1158.
10. Gillman J.C., A manual of soil fungi, Oxford and IBH Publishing Company, Calcutta., 1959 , pp 215.

11. Gillman J.C., A manual of soil fungi, Biotech books, New Delhi., 1998, 235.
12. Glicksman, M., Food hydrocolloids. Vol II. CRC Press Inc, Boca Raton, Florida 1983.
13. Jeffrey L.S.H., Son R. and Tosiah S., Preliminary screening of endophytic fungi isolated from medicinal plants at MARDI Sessang, Sarawak for their bioactivity. *Journal of Tropical Agriculture and Food. Science* 2008, **36:** 121–126.
14. Karthik, A.; Gobalakrishnan, S.; Jaffar Hussain, A. and Muthezhilan, R., Efficacy of Bacteriocin from *Lactobacillus* Sp. (AMET 1506) as a Biopreservative for Seafood's Under Different Storage Temperature Conditions. *Journal of Modern Biotechnology* 2013; **2:** 59–65.
15. Mishra, P.C.; Jayasankar. R. and Seema.C., Yield and quality of carrageenan from *Kappaphycus alvarezii* subjected to different physical and chemical treatments. *Seaweed Research Utilization* 2006; **28** (1) : 113-117.
16. Mochtar, A.H.; Parawansa,I.; Saleh, S.; Ali, Jusoff, K.; Reta, Rezekie, Astuti, Nasruddin Azis,S.D.; Muchdar, A.; Palad, M.S.; Hikma, Nonci, M.; Kasmawati. and Nirwana. Effects of Harvest Age of Seaweed on Carragenan Yield and Gel Strength. *World Applied Sciences Journal* 2013; **26:** 13-16.
17. Sadaf Jahangeer, Nazia Khan, Saman Jahangeer, Muhammad Sohali, Saleem Shahzad, Aqeel Ahmad. and Shakeel Ahmed Khan., Screening and characterization of fungal cellulases isolated from the native environmental source. *Pakistan Journal of Botany* 2005; **37:** 739-748.
18. Sallam, K.I., Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. *Food Control* 2007; **18:** 566–575.
19. Sathyavrathan, P. and Krithika, S., Comparison of Cellulase Production In *Trichoderma reesei* (NCIM - 1052) and *Aspergillus niger* (NRRL - 322); Media Optimization and Enzyme Characterization of Cellulase from with Lyophilization. *International Journal of ChemTech Research* 2013, 5: 554.
20. Sunil, M., Antimicrobial Food Packaging to Enhance Food Safety: Current Developments and Future Challenges. *J Food Process Technol* 2012; **3**: 103.
21. Sri Lakshmi, A. and Narasimha. G., Production of cellulases by fungal cultures isolated from forest litter soil. *Annals of Forest Research* 2012; **55**: 85-92,
22. Soovendran, A., Development of high yielding carragenan extraction method from *Eucheuma Cotonii* using cellulase and *Aspergillus niger. Seminar Kimia Bersama UKM-ITB Proceedings* 2009.
23. Subramanian C.V., Hyphomycetes – An Account of Indian species except Cercosporiae, ICAR, New Delhi., 1971, 206.
24. Subbulakshmi, G.K.; Thalavaipandian, A.; Ramesh, V.; Bagyalakshmi. and Rajendran A., Bioactive endophytic fungal isolates of *Biota orientalis(L) Endl Pinusexcelsawall and thujaoccidentalis, International journal of Advanced Life Sciences* 2012; **54:** 278-254.
25. Vicentini, N.M., Preparation and caracterization of edible films based on cassava starch for use post harvest. *Ph.D. Thesis, State University, Julio Mesquita Filho* 2003.
26. Weber, C.J.; Haugaard, V.; Festersen, R. and Bertelsen, G., Production and applications of bio-based packaging materials for the food industry. *Food Additives and Contaminants* 2002; **19:** 172-177.
27. Zinash, A.; Workneh, T.S. and Woldetsadik, K. Effect of accessions on the chemical quality of fresh pumpkin. *African Journal of Biotechnology* 2013; **12:** 7092-7098.