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SHORT REPORTS

Utilization of Shrimp waste as a novel media for marine bacteria isolation

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Introduction

Marine organisms such as sponges, tunicates, corals, seaweeds and Algae etc. that harbour symbiotic microorganisms contributed for the discovery of novel bioactive compounds. Progress of marine-derived bioactive compounds is gradually increasing for the last 20 years. Nearly 15,000 marine bioactive compounds were discovered by the year 2010 and resulted in anticancer drugs such as Cytarabine, Eribulin mesylate and analgesic compound Ziconotide. Thus, the Marine environment was proved as a pivotal source for diverse bioactive compounds such as antifungal, antiviral, antibacterial and anticancer, etc. (Mehbub et al. 2014). Generally, Growth of microorganism requires carbon source, nitrogen source, pH, osmotic conditions, trace elements and temperature. The marine bacterial community has been isolated by using Zobell marine agar (Zobell 1941) and it needs seawater as a stringent requirement for its growth and as diluents of the isolation medium (Zobell and Upham 1944). A practical barrier such as cultural conditions, high concentration of media constituents and combination of substrates has been associated with marine bacteria isolation. Bacteria in a marine habitat thrive at low concentration of organic substrate. Ultimately, marine bacteria growing in laboratory settings needs an appropriate nutrient source and sufficient incubation time to grow in case of slow-growing microbe (Joint et al. 2010). Archea from marine habitats such as deep sea, euphotic zone and marine sediments reported earlier as an example for the uncultured microbial population (Santoro et al. 2019; Danovaro et al. 2016). Recent reports revealed that methodological innovations and alteration in media paved the way to grow the uncultured bacteria (Steen et al. 2019). Zobell marine agar and nutrient broth with 50% aged seawater were commonly used as marine bacteria growth medium (Inbaneson et. al. 2012) containing all the essential nutrients. Shrimp is highly utilised seafood and 40 to 50% of the raw material (head, tail and shell) was accumulated as waste material in most of the species and 150,000 tonnes of shrimp waste was generated annually in India (Prameela et al. 2017). Shrimp waste has a good source of animal protein with other biomaterials such as chitin, lipids, pigments, amino acids and trace elements (Mao et al. 2017). Chemical treatment for the recovery of biomaterials such as chitin, protein and carotenoids from shrimp waste resulted in a toxic effluent generation. Shrimp waste in the environment putrefied in tropical climatic condition and leading to biogenic amines synthesis (Kandra et al. 2011; Mao et al. 2017). Shrimp waste was digested with microbial proteases to prepare functional food additive (Dey et al. 2014). Chitin was extracted from shrimp waste using Cofermentation using *Bacillus licheniformis* and *Gluconobacter oxidans* (Liu et al. 2014) and by fermentation with *Bacillus licheniformis* (Guo et al. 2019). Bioadsorbent material such as Hydrochar from shrimp waste was used for dye removal (He et al. 2020). Protein-rich food such as Tilapia fish feed was prepared from shrimp waste (Ximenes et al. 2019). Protease and antioxidant material from shrimp waste (Kumar et al. 2014), Biofuel feedstock from shrimp waste were obtained (Srimongkol et al. 2019).

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Commercial media such as Artificial Sea Water Salts broth, Marine broth 2216 and Zobell marine broth, widely used for culturing marine bacteria are expensive. Marine bacteria growth media has peptone, yeast extract except for carbohydrate source with trace elements similar to that of seawater for culturing marine bacteria (Vazquez et al. 2004). Synthetic media for culturing marine bacteria in lab-scale may not require more financial investment. But, any marine bacteria which has potential in synthesizing novel bioactive compounds need to be grown in low cost/ economical media for mass scale cultivation. So, The present study made an attempt to prepare culture media for marine bacteria from cost-effective raw material like shrimp waste. As it is a waste material, available in large quantity even for large scale production. Moreover, Shrimp waste is accumulated in large quantity lead to environmental pollution and solid waste disposal issue. Thus, conversion of waste material into microbiological media was explored and could be proposed as an alternative media for growth of marine bacteria.

Materials and methods

Marine soil was collected from Pondicherry coastal region and immediately transferred to a sterile plastic container and stored at 4 °C. Marine soil was serially diluted (10^{-1} to 10^{-7}) as per standard microbiological procedure and inoculated using spread plate method. Shrimp waste was collected from the fish market in Pondicherry and finely ground with a blender to make paste. Shrimp paste was dried under shadow for several days and checked for the absence of moisture content. Further, it was converted into powder form. Shrimp waste powder was added in double distilled water at 1% concentration and heated for 100 °C for 5–10 min. The solution was filtered using Cheese cloth or filter to get supernatant. Further, it was added with an equal volume of 50% aged seawater and 2% agar. The nutrient broth in 50% aged seawater with 2% agar was used for comparison. Both the media was poured into sterilized petri plates and allowed for solidification. 100 µl from respective soil dilution was added in the plate and spread using sterile plastic L spreader. For each dilution, duplicate plates were used with control and experiment was done three times. Plates were incubated at room temperature for 24 h and the colony was counted.

Powdered shrimp waste & shrimp waste supernatant were analyzed for proximate nutrient composition at Indian Institute of Food Processing Technology (FSSAI Referral & NABL Accredited laboratory), Thanjavur, Tamilnadu. To examine the bioactive potential, Shrimp waste media was tested in marine sponge-associated bacteria for its pupicidal activity. Seed culture was grown in zobell marine broth for overnight and 2% inoculum was transferred to 100 ml shrimp waste media with a carbon source (dilution of 1% Shrimp waste media with carbon source below 3%). Flask was kept under incubation at 250 rpm for 4-5 days. Afterwards, culture broth was used for preliminary bioassay against 20 nos of field collected *Culex sp* in 100 ml of chlorine free tap water in each bioassay cup. Pupal mortality or moribund pupae was observed after 24 h by counting live pupae in the bioassay cups. Three different bioassay experiments were conducted with two replicates and percentage mortality was calculated.

Results and discussion

Figure 1 was shown to visualize the growth of marine bacteria in respective serial dilution (10^{-3}) was plated in two different media namely nutrient agar with 50% aged seawater and shrimp waste agar with 50% aged seawater. Viable cell count represented in Table 1 for both the media was almost similar in all the dilutions. Viable cell count in shrimp waste media revealed that it supported the growth of marine bacteria with reproducible results. Table 2 revealed the proximate nutrition analysis of shrimp waste and shrimp waste supernatant used for the comparison with an earlier report by Henson et al. (2016). 1% shrimp waste media was prepared by using 10 g of shrimp waste powder (1.45 g carbon and 3.83 g nitrogen)/litre. From the prepared above solution, 500 ml was used for the media preparation and was made up to 1 L using aged sea water. Hence, the final media contains (0.725 g carbon and 1.91 g nitrogen/litre) and trace elements were supplied by 50% aged seawater. Similarly, it was calculated for shrimp waste supernatant and found to be the (0.040 g carbon/litre) from the present study Further, artificial seawater media used for the growth of most of the marine bacteria containing a minimal amount of carbon (0.0008 g/litre) and nitrogen (0.009 g/litre) was reported by Henson et al. (2016). The present study reported that shrimp waste had a good amount of nitrogen and carbon source whereas Henson et al. (2016) stated that minimal amount of carbon (0.028 g/litre) & Nitrogen (0.0005 g/litre) in seawater Inorganic ion requirement of the marine bacteria was supplied by the sea water used in the preparation of nutrient agar and shrimp waste agar and it is corroborated with earlier findings of Zobell and upham (1944).

Sponge homogenate was grown in a minimal media (1.8% agar with 80 µm filtered seawater) supported the growth of many bacterial phylum such as alphaproteobacteria and bacteroidetes were grown in the same minimal media using Diffusion Growth Chamber (DGC) method in marine sponge *Rhabdastrella globostellata* (Steinert et al. 2014). Screening of glutaminase enzyme-producing bacteria from marine habitat was grown in minimal media containing seawater (Iyer et al. 2009). Thus, it revealed that the minimal media also supported the growth of marine bacteria that mimics the

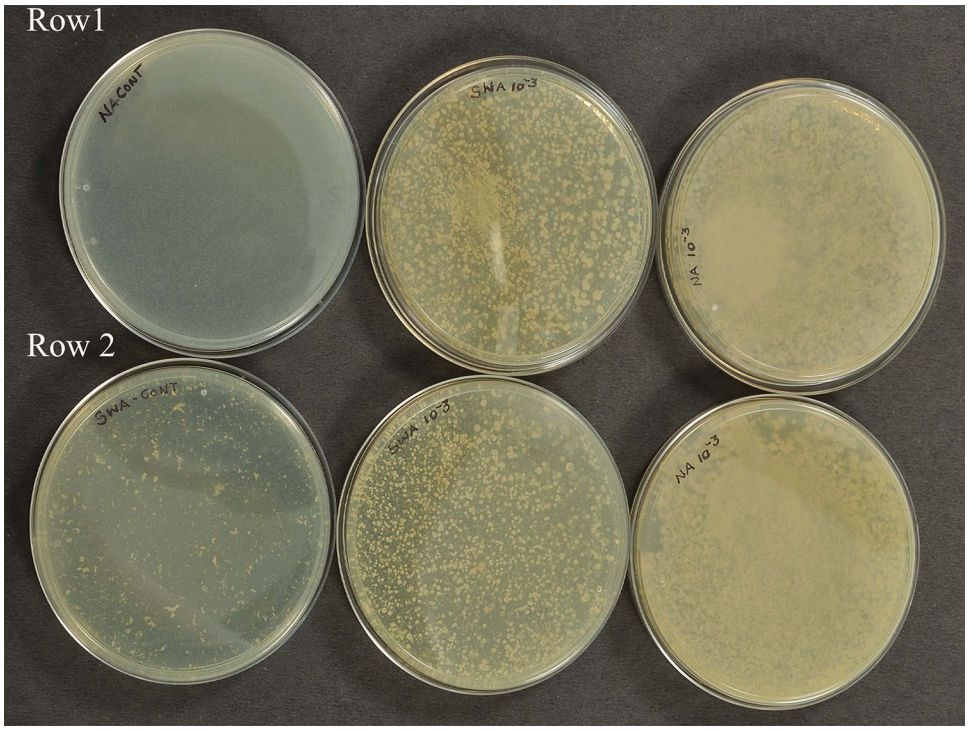

Fig. 1 Picture showing the colonies of marine bacteria in nutrient agar and shrimp waste agar in 10^{-3} dilution. Row 1: from right to left: nutrient agar (control); shrimp waste agar, nutrient agar. Row 2: from right to left: shrimp waste agar (control); shrimp waste agar, nutrient agar

Table 1 Number of colonies observed in nutrient agar and shrimp waste agar

Dilution	Nutrient Agar (NA)	Shrimp Waste agar (SWA)
10^{-3}	TNTC	TNTC
10^{-4}	TNTC	TNTC
10^{-5}	100 (81.9%)	99 (87.6%)
10^{-6}	16 (13.1%)	11 (9.7%)
10^{-7}	6 (4.9%)	3 (2.7%)
Total of all replicates	122	113

Table 2 Proximate nutrition analysis of Shrimp waste and Shrimp waste supernatant

S. No	Parameters analyzed	Shrimp Waste (g/100 g)	Shrimp waste Supernatant (g/100 g)
1	Moisture[a]	15.30[1]	98.98[2]
2	Carbohydrate[b]	14.59	0.81
3	Protein[c]	38.32[1]	Not detected[2]
4	Fat[a]	2.68[1]	Not detected[3]
5	Crude fiber[d]	4.16[1]	Not detected[2]
6	Ash[a]	24.95[1]	0.21[4]
7	Energy[e] (Kcal)	235.76	3.24Kcal

[a1]AOAC, 20th edn. (2016) 945.38, Cha, 32.2.01, Vol II, pg: 46, [a2] AOAC, 21s[t] edn, (2019) 925.10;Cha,32.1.03;Vol II; Pg:1, [a3]AOAC,21st edn, 2019; 2003.05; Cha 4.5.05; Vol I; Pg 41, [a4] AOAC, 21st Edn, 2019,923.03; Cha32.1.05; Vol II, Pg 2

[b]Sadasivam S et. al., (2005) Biochemical methods, revised second edition. pg. 8–9

[c1]AOAC, 20th edn. (2016) 979.09, Cha, 32.2.03, Vol II, pg:52, [c2] AOAC, 21st edn. 2019, 984.13, Cha, 4.2.09, Vol I,Pg:31

[d1]AOAC, 20th edn. (2016) 920.86; Cha 32.1.15; Vol II; pg:5, [d2] AOAC, 21st edn. 2019; 962.09; Cha4.6.01; Vol I; Pg 44

[e]Vetter JL, Melran EM (2007) Food labelling—Requirements for FDA regulated products, AIB International, Manhattan

natural environment. So, the present study attempted with the cost-effective Shrimp waste medium for marine bacteria isolation using standard plating technique. Still, shrimp waste media in this study could be modified by increasing the concentration to meet the nutritive content of synthetic media as well. In this connection, shrimp waste agar supported the growth of marine bacteria almost identical to nutrient agar. Hence, it acted as a basal medium to support the growth of marine bacteria.

Cost of making shrimp waste agar for 1litre is US$4 and the estimated cost of Zobell marine agar and nutrient agar with 50% aged seawater would be US$14.5 and US$7.5. shrimp waste agar used in this study is 3.6 times and 1.8 times cheaper than zobell marine agar and nutrient agar respectively. Agar is the one component in shrimp waste agar contributing a major portion of the total cost. Cost analysis (per litre) in culturing of marine bacteria using zobell marine broth and nutrient broth were estimated as US$10.5 and US$3.5 whereas the negligible cost of US$0.002 may be required for shrimp waste-based broth (Table 3).

Table 3 Cost analysis for Shrimp waste media with various synthetic media

S. No	Culture media	Quantity of media for 1 L preparation (g/Litre)	Total cost of media in US$	Cost difference in ratio between shrimp waste and other media
1	Zobell Marine agar	55.25 g	US$14.5	3.6
2	Nutrient Broth prepared in 50% aged sea water + Agar	13 g + 20 g agar	US$3.5 + US$4 = US$7.5	1.8
3	Shrimp Waste* + Agar	10g* + 20 g agar	US$0.002 + US$4 = US$4.002	
4	Zobell marine broth	40.25 g	US$10.5	
5	Nutrient broth	13 g	US$3.5	
6	Shrimp waste	10 g	US$0.002	Negligible
	*Shrimp waste preparation includes collection, transportation, Shadow drying and grinding for laboratory scale	*Used for making 1 L shrimp waste & each 500 ml shrimp waste used to make 1 L preparation		

Sponges in marine environment possess a diversified group of microorganisms and it is proved as the richest source of bioactive compounds among marine living organisms. 30% of marine natural products and nearly 200 products every year were derived sponges and it has huge applications in the health care sector. (Mehbub et al. 2014). Sponges have a good association with bacteria as well as archea and acquired by sponge host by host geography and the environment. Studies indicated that Archea was involved in ammonia oxidation and holobiont functioning in sponge host. (Turon et al. 2020) Similarly, bacteria in sponge host also involved in nutrient uptake and defence etc.. Archae bacteria in marine sedimentary regions are involved in nitrogen, carbon and sulphur cycles and it was reported for the synthesis of halophilic enzymes, pigments, bioplastics and a good candidate for bioprospecting (Santoro et al. 2019, Antunes et al. 2016).

The present study focused on the symbiont bacteria associated with the sponge *Stylissa carteri.* Sponge *Stylissa carteri* was collected from Thondi in palk strait region of Tamilnadu and symbiont bacteria was isolated (Inbaneson et al. 2012) and cryopreserved in 15% glycerol. Further, Shrimp waste media was tested by growing marine sponge *Stylissa carteri* associated symbiont bacteria to reveal its mosquitocidal activity and was grown in shrimp waste media supplemented with carbon source and showed 81.6% mosquito pupicidal activity. Stephenson et al. (1976) reported that shrimp head digest supported the growth of bacteria and fungi in 0.5% concentration whereas the present study revealed that shrimp waste could be used as growth media for sponge symbiont bacteria and reported for mosquito pupicidal activity for the first time. Further investigation in the optimization of shrimp waste media with other nutrients & the bioactive compound characterization for mosquitocidal activity is under progress (Unpublished data). Further activity of marine bacterial isolate using shrimp media (Biosurfactant production) needs to be investigated.

Marine bacteria in the marine habitat considered a good resource for bioactive compounds. Marine metabolites could be unique in terms of structural novelty, complexity and diversity (Lindequist et al. 2016). For any marine bacteria, isolation and bioactive compounds synthesis need a media that suits the growth of marine bacteria. In this connection, shrimp waste is a novel media with all the essential nutrients and remains unexplored in the conversion of waste into a bacteriological growth media. Preparation of this media needs only shrimp waste material and simple technique was only involved in further processing into growth media. Thus, the media will definitely be a good source for the cultivation of marine bacteria at low cost and this can be extended to Industrial scale cultivation with optimization. Further study is recommended to monitor the microbial flora in marine soil at different sites and various seasons for validation of this result. Hence, bioconversion of this shrimp waste into growth media will be useful in the isolation of marine bacteria and initiate the bioprospecting of marine bioactive compounds. Further, optimization of this media with OSMAC (One Strain Many Compounds) approach will facilitate the discovery of novel metabolites.

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Author contributions AM conceived the concept and conducted experimental work, manuscript writing and technical assistance performed by KD. Overall supervision and manuscript writing guidance was done by SR and GS.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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