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Evaluation of locally-available agricultural and industrial waste materials as effective carriers for bacterial inocula in freshwater bioremediation

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A B S T R A C T

The pollution of freshwater is a pressing global environmental concern, necessitating effective management strategies for polluted aquatic environments. Bioremediation has emerged as a highly promising environmentally friendly approach. However, the selection of suitable candidates capable of effectively degrading or removing pollutants remains a challenging task. The introduction of live candidates, particularly bacteria, into natural environments also poses its own set of difficulties. To address these challenges, immobilizing bacteria within carrier materials has emerged as a leading option. In this study, we meticulously assessed the suitability of four locally-available and low-cost agricultural and industrial waste materials as carriers to transport bacteria into water bodies. The selection criteria encompassed bacteria immobilization capacity, viability, and the resulting water quality after treatment. In order to facilitate comparison, the widely-used sodium alginate was included as a benchmark, and *Escherichia* coli was employed as the model bacterial inoculum. Our findings revealed that alkaline pre-treatment of corn husk, rice husk, rice straw, and sugarcane bagasse significantly enhanced the bacteria immobilization capacity of these materials. Notably, the viability of bacteria in carrier materials, including sodium alginate, exhibited remarkable resilience, with a count of 107 CFU/g material even after 49 days of storage at room temperature. Moreover, upon determining the quality parameters of the receiving water, the introduction of rice husk and sodium alginate materials demonstrated no significant adverse impact. The quality parameters were well within the acceptable range defined by the World Health Organization standards for drinking water and the Sri Lankan ambient water quality standards for various purposes. Based on the overall performance evaluation, we advocate for the application of rice husk and sodium alginate as superior carriers for delivering bacterial inocula to aquatic environments, particularly in polluted water bodies targeted for bioremediation efforts. Nonetheless, we recommend the collection of carrier materials only after the establishment of bio inoculum in the receiving water, as a precautionary measure to minimize any potential impact on the chemical oxygen demand of the water.

1. Introduction

Freshwater bodies around the globe are highly susceptible to contamination by a diverse array of pollutants, including agrochemicals, industrial discharges, and domestic wastewater, which find their way into watercourses through direct or indirect means (Amoatey and Baawain, 2019). Moreover, the pollution of freshwaters with biological agents, such as pathogenic microorganisms and toxic byproducts like cyanotoxins produced by cyanobacteria, has emerged as a pressing environmental concern on a global scale. The pollution of freshwater poses a significant threat to the well-being of aquatic organisms and, in turn, poses risks to human health. Conventional physical and chemical remediation methods, currently in practice, are often costly and can lead to the generation of secondary pollutants,

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exacerbating the complexity of the problem. Consequently, the implementation of bioremediation has garnered considerable importance as an environmentally-friendly approach to restoring polluted aquatic environments (Ojuederie and Babalola, 2017; Skinder et al., 2020). Bioremediation with heterotrophic bacteria is considered more effective over other organisms due to some inherent abilities of bacteria such as wide host range, adaptability to variations in environmental conditions and host responses, efficient multiplication, ability to search for or trap prey, and survival at low prey densities by switching or adapted to other food sources (Daft et al., 1985). Usually, selected bacterial strains or consortia are introduced to the water as a liquid culture inoculum. In this method, bacterial inoculum may not be confined to the targeted sites to be remediated as bacterial cells may disperse horizontally and vertically in the water column and also may not survive due to competition with native microflora (Nunal et al., 2014). Hence, the utilization of immobilized bacterial inocula offers enhanced retention of bacteria at the intended site and prolongs the operational lifespan. Furthermore, the constrained mobility of bacterial cells or the secreted enzymes and other metabolites may contribute to sustaining efficient pollutant degradation (Li et al., 2013). Also, immobilization provides enhanced stability and survival of bacteria (Cassidy et al., 1996). The natural ability of bacteria to form biofilms on the surfaces of various materials is the key characteristic feature necessary for immobilization (Bayat et al., 2015).

In addition to the identification of suitable bacterial candidates, the selection of appropriate carrier materials for the immobilization of bacteria represents the most challenging stage in the development of a successful bioremediation approach. Equally important is the consideration of the nature of the intended application (whether *in-situ* or *ex-situ*), the type of pollution being targeted, and the specific properties of the immobilized microorganisms (Dzionek et al., 2016). A carrier material is an abiotic substrate (solid, liquid, or gel) that is used in the formulation process (Bashan, 1998). An ideal carrier should be insoluble, non-toxic to either immobilized organism or material and to the environment, easily accessible, inexpensive, biodegradable, stable, and suitable for regeneration (Martins et al., 2013). Further, if carriers are to be used for adsorption or binding on the surface, they should have high porosity and surface-to-volume ratio to provide sufficient attachment sites (Li et al., 2017). Generally, carriers are classified as organic or inorganic, natural or synthetic materials. Widely used natural organic carriers include alginate, carrageenan, chitosan, sawdust, straw, charcoal, plant fibers, corncob, bagasse, husks of sunflower seeds, corn, and rice (Obuekwe and Al-Muttawa, 2001). In Sri Lanka, a large portion of agricultural residues with high lignocellulosic content are wasted daily by burning or burying. In addition, tons of waste bio-mass are accumulating daily in industries like sugarcane, rubber, rice, coconut, and oil palm. Further, the expected paddy production per year is 3.8 million metric tons and it is estimated that from 1 ton of rice, 292kg of rice straw and 220kg of rice husk are produced (Siriwardena and Subasinghe, 2020). Burning or disposal of rice straw in the paddy field is the general practice of farmers in rural areas (Arachchige et al., 2020). Besides paddy, corn farms account for the second largest land extent where, around 30,000 h of land is cultivated annually in Sri Lanka (Jayaratne et al., 2020). In the context of domestic corn production, husks are often discarded without being utilized for any secondary purposes (Wijesingha and Perera, 2017). In the sugarcane industry, it is observed that approximately 3 tons of wet bagasse are generated for every 10 tons of sugarcane crushed (Arachchige and Sakuna, 2019). A fraction of this bagasse is utilized for steam generation and biofertilizer production, while the remaining portion is discarded (Bandara, 2015).

Lignocellulosic waste materials have garnered attention as promising sources of bio carriers (Nunal et al., 2014), owing to their cost-effectiveness, eco-friendliness, renewable nature, and non-toxicity (Zhang et al., 2019). Moreover, the presence of lignin, cellulose, and hemicellulose within these materials offers potential as nutrient sources to support bacterial growth and survival. However, due to the highly condensed structural organization of lignin and cellulose, pre-treatment procedures are often necessary to loosen the structure and facilitate accessibility for immobilized organisms (Santos et al., 2008). Among these pre-treatments, alkaline treatment using NaOH has been reported as highly effective, particularly for hardwood and agricultural residues with relatively low lignin contents (10–18 %) (Taherdanak and Zilouei, 2014).

In Sri Lanka, enough attention has not been paid to using agricultural waste materials as carriers of bacteria in field applications. There are only a few published reports available. One such study attempted to amend compost with three different *Trichoderma* spp. to control diseases caused by *Fusarium oxysporum* and *Rhizoctonia solani* (Hewavitharana et al., 2018). Two other studies showed the potential application of bacteria for the bioremediation of oil-contaminated soil and water (Gamage et al., 2021; Wijesooriya et al., 2022). However, to our knowledge, bioremediation of polluted water using immobilized bacterial formulations in the Sri Lankan context has not been tested to date.

In this study, we selected readily available agricultural and industrial waste materials to test their suitability as carrier materials to introduce heterotrophic bacteria into freshwaters for the purpose of bioremediation of polluted water. For comparison, a widely used bacterial carrier in soil applications, sodium alginate was used. As the bacterial inoculum, a model bacterial species, *Escherichia coli* was used. Bacteria immobilization efficiency, viability of bacteria, and the effect of carrier material on the water quality were used as criteria for evaluation.

2. Materials and methods

2.1. Preparation of carrier matrix

Four types of agricultural and industrial waste materials; sugarcane bagasse (at 50% moisture content), rice husk, rice straw, and corn husk were tested for the selection of suitable carrier materials of bacterial inoculants. All materials were washed with tap water to remove adhering soil and dust particles and oven-dried to obtain constant weight at 100°C. A fine powder of each material was obtained by grinding with a household grinder followed by passing through a 0.42mm sieve (BSS 40) and oven drying for 3 h at 100°C.

2.2. Alkaline pre-treatment

Materials were pre-treated with an alkaline solution to loosen the compact lignocellulosic structure in the cell wall and consequently enhance the efficiency of bacteria immobilization. A hundred grams of fine powder of each material was pre-treated with NaOH following slight modifications to the method described by Santos et al. (2008). Briefly, 1.66L of 0.5M NaOH was added into 100g of powdered material and kept shaking for 24 h at 150 rpm in a horizontal orbital shaker (Stuart SSL1, UK). Alkali-treated materials were collected by filtration (11µm, Whatman No 1) and washed with distilled water until the filtrate become colorless. The collected material was oven dried for 3 h at 100°C and autoclaved in separate 250mL Erlenmeyer flasks, each containing 10g of material.

2.3. Preparation of bacterial inoculum

A strain of *Escherichia coli*, obtained from the culture collection of the Department of Botany, University of Ruhuna, Sri Lanka was used as the model bacterial strain. Bacterial inoculum was prepared from a culture grown in nutrient broth for 16 h at room temperature (approximately 30°C) and constant shaking at 120rpm (Stuart SSL1, UK). Cells were harvested by centrifugation at 5000rpm for 5 minutes (Xiangzhi TGL21M, China) and washed twice with sterilized distilled water. The cell pellet was resuspended in 300 mL of 0.1M potassium phosphate buffer (pH=6.8±0.1). The optical density (OD) of the suspension was adjusted to 1 x 10^8 cell/mL.

2.4. Immobilization of bacteria into carrier materials

Seventy-five milliliters of bacteria suspensions at 1 x 10^8 cell/mL density were added into flasks containing 10g of autoclaved material and incubated for 24 h, at room temperature. Flasks were provided with constant shaking at 150rpm in a horizontal orbital shaker (Stuart SSL1, UK) to promote bacteria immobilization on the surface of carrier materials. Materials with immobilized bacteria were collected by filtering through sterilized Whatman No 1 filter (11µm) papers and air dried for 1 to 2 h under a laminar-flow hood. Bacteria-immobilized dried materials were transferred into sterilized screw-capped 250mL flasks and stored for 60 days at room temperature.

To assess the efficacy of bacterial immobilization within carrier materials, the widely-used bacterial carrier material, sodium alginate, was employed using a previously published procedure with slight modifications (Lotfipour et al., 2012). In summary, a slurry containing 4% (w/v) sodium alginate was prepared, autoclaved, and subsequently cooled to room temperature. A volume of 25 milliliters of bacterial inoculum, with a density of 1 x 108 cells/mL, was aseptically homogenized with 25 milliliters of 4% (w/v) sodium alginate, resulting in a final sodium alginate concentration of 2% (w/v). The mixture was stirred for 15 minutes at room temperature using a magnetic stirrer to facilitate bacteria immobilization.

The bacteria-sodium alginate homogenate was then carefully introduced into a sterile 5mL syringe and manually dispensed, at an average speed of 70 droppings per minute, into 125 milliliters of a 2.5% (w/v) $CaCl_2$ solution while maintaining continuous stirring. This process led to the formation of beads with a uniform size, where bacteria were anticipated to be immobilized both on the surface and within the matrix of the beads (encapsulated). The resulting beads were allowed to harden for 1 hour and subsequently harvested by filtration through Whatman No 1 filter paper (11 µm). After filtration, the beads were washed with sterilized distilled water to remove any remaining $CaCl_2$ and loosely attached bacterial cells. Finally, the beads were air-dried in an aseptic environment for 1 hour under a laminar-flow hood.

The dried bacteria-sodium alginate beads were then transferred into sterilized screw-capped 250mL flasks and stored at room temperature for a duration of 60 days.

2.5. Determination of viability of bacteria

The viability of bacteria immobilized within the materials was assessed over an 8-week period through weekly plate assays. A 10-fold dilution series was prepared from 1g of the bacteria-immobilized materials in 10mL of sterilized distilled water. Simultaneously, the viability of bacteria immobilized in sodium alginate was also determined using a 10-fold dilution series. To accomplish this, 1g of bacteria-immobilized beads was crushed to obtain a homogenate in 10mL of sterilized distilled water using a sterilized motor and pestle. Subsequently, 100 microliters of selected dilutions were plated onto nutrient agar medium with three replicates per dilution.

The quantification of Colony Forming Units (CFU) was performed using a colony counter (Rocker, Taiwan). Furthermore, colony morphology assessment and Gram staining were conducted to verify the absence of any contaminating bacteria-immobilized carrier materials.

2.6. Scanning electron microscopy

Confirmation of bacteria immobilization within the tested carrier materials and sodium alginate was achieved by capturing scanning electron microscopic (SEM) images. This approach allowed for visualizing the microstructure of both the alkaline-treated and untreated carrier materials, as well as the sodium alginate while observing the attachment of bacteria. Prior to imaging, samples of the carrier materials and sodium alginate beads were meticulously prepared by mounting them on aluminum stubs using conductive carbon tape. Subsequently, a thin layer of gold was sputtered onto the samples under vacuum conditions at 10mA for 90 seconds, using the Sc7620 sputter coating system.

The prepared samples were then subjected to SEM imaging at specific parameters, including a working distance of 14.5mm, an accelerating voltage of 10.00kV, and a magnification level of 5000X, utilizing the EVO 18 microscope by Carl Zeiss AG, Germany.

2.7. Evaluation of the effect of carrier material on the water quality

A straightforward experimental setup was devised to assess the impact of carrier materials on water quality by monitoring key water quality parameters. In brief, 4g of each alkali-treated individual carrier material was enclosed within sachets (4 x 8 cm) made of cotton fabric, under strict aseptic conditions. These sachets were then partially submerged in the water column by being suspended from the lids of plastic bottles containing 1L of dechlorinated tap water. To ensure proper air circulation, insect-proof netting was affixed to the container lids. As a control, 1L of dechlorinated tap water was used without any carrier material. The entire experimental setup was maintained at room temperature for a duration of 7 weeks.

Water quality parameters, pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), salinity, turbidity, temperature, and nitrate nitrogen (NO_3^-) were measured using a multi-parameter water quality meter (HI9829-HANNA, Romania). Chemical oxygen demand (COD) was measured following the reactor digestion method described by the US-Environmental Protection Agency (US-EPA, 1983), where 2mL of water from each treatment and control were separately added into COD digestion reagent vials with 20-1500mg/L detection range (Hatch, USA) and digested at 150°C for 120 min. The COD was measured using COD Set-Up (MD 600 Lovibond, Germany). Readings were taken at weekly intervals for a period of 7 weeks.

To investigate the impact of bacteria immobilization on the COD in water, an identical experimental setup was employed, employing sachets containing 4g of carrier materials with immobilized bacteria. The COD levels were measured in the 1st, 4th, and 8th weeks after the inoculation of the carrier materials with bacteria.

2.8. Statistical analysis

Statistical analyses were performed by using a standard statistical software package MINITAB version 17. Data were tested for normality prior to the analysis. The results of the measured parameters were reported as the arithmetic mean of three independent measurements. One-way analysis of variance (one-way ANOVA) followed by Turkey's post hoc tests was applied to determine significant differences among means. A paired t-test was also applied in order to compare the water quality in water-containing carrier materials with that of sodium alginate-containing water. The significant difference of all analyses was defined at $p<0.05$.

3. Results and discussion

The transportation of bio inoculants from the laboratory to natural environments poses one of the most formidable challenges in bacteria-mediated bioremediation of polluted habitats. Hence, the selection of appropriate carrier materials becomes a crucial aspect of this process. Key considerations often encompass the availability and cost-effectiveness of the materials. In this study, we sought to evaluate the suitability of four readily available and cost-effective waste materials for delivering a bacterial inoculum into freshwater, using *E.* coli as the model bacterial strain. The selection criteria were based on three key parameters: the efficiency of bacterial immobilization on the carrier material, the viability of the bacteria, and the potential impact of the carrier material on the water quality in the receiving environment.

3.1. Enhanced bacteria immobilization in alkaline pre-treated material

SEM images were utilized to visualize the attachment of bacteria on the surface materials. It was observed that bacteria immobilization was significantly enhanced in materials treated with the alkaline solution. The pre-treated materials exhibited a notably higher density of attached bacteria compared to the non-treated materials (Fig. 1).

The lignocellulosic biomass found in plant materials predominantly consists of cellulose, hemicellulose, and lignin, which are interconnected by covalent hydrogen bonds, resulting in a highly recalcitrant structure. The majority of bacteria are unable to hydrolyze lignin (Haider et al., 1978).

Consequently, the lignin cross-linking present in the cell wall obstructs the free access of bacterial cells and their hydrolyzing enzymes to reach the hydrolyzable materials within the cell wall, such as cellulose and hemicellulose.

However, when the materials are treated with an alkaline solution, the ester bonds between lignin and hemicellulose, along with other components, undergo disintegration due to saponification. This leads to an increase in the porosity of the lignocellulosic biomass, facilitating unrestricted access for bacterial enzymes to reach the hydrolyzable biomass and also providing more surface area for bacterial attachment (Bassan et al., 2016). This presumed mechanism accounts for the higher density of attached bacteria observed on the surface of alkali-treated carrier materials compared to the non-treated ones.

Based on these results, the subsequent experiments utilized alkaline pre-treated materials due to their superior bacterial attachment properties.

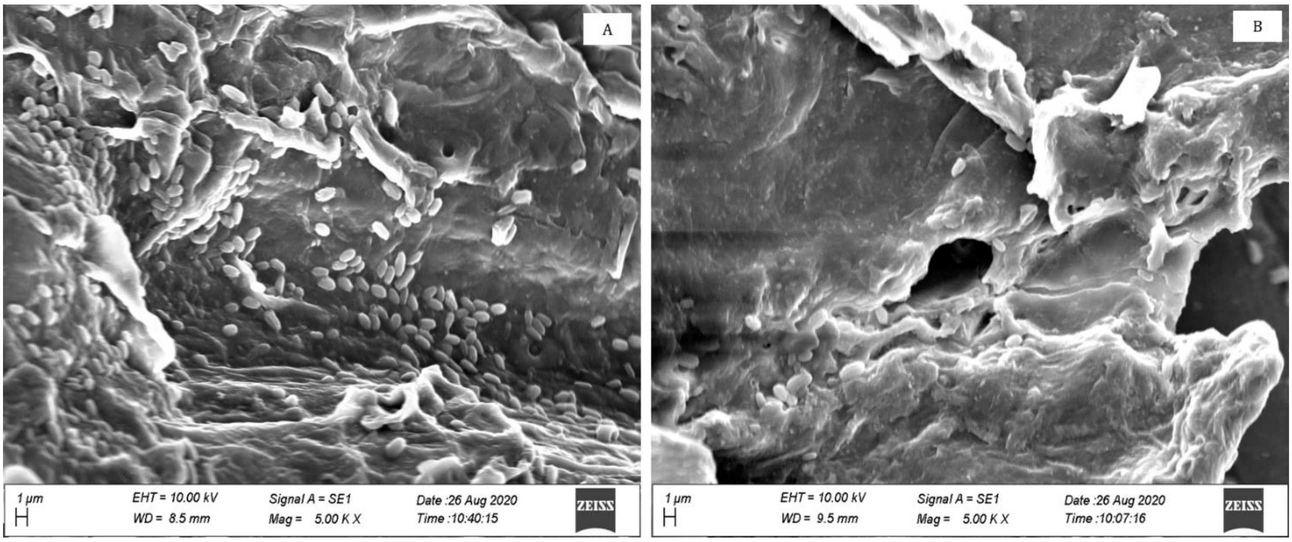

Fig. 1: SEM images showing enhanced bacteria immobilization in alkali-treated sugarcane bagasse (A) compared to non-treated material (B)

3.2. Viability of bacteria in immobilized carrier materials

While SEM images have provided evidence of bacteria physically attaching to the surface of carrier materials, ensuring their viability within the carriers is a critical aspect. The maintenance of sufficient bacterial viability over time is a desirable trait for an effective bacterial carrier, as viable bacterial cells serve as the inoculum source in the target aquatic environment. The results indicated that all tested materials exhibited a high level of bacteria viability (Table 1). Notably, the initial bacteria count (1 day after immobilization) in all four tested carrier materials was approximately 30-90 times higher than that of sodium alginate. This disparity could be attributed to the finely ground carrier materials, which possess a high surface-to-volume ratio, providing more attachment sites for bacteria compared to the 2mm diameter sodium alginate beads.

After 49 days, the bacterial count reached a magnitude of 107 CFU/g in all carrier materials, including sodium alginate. These results collectively confirm that all tested carrier materials were capable of maintaining fairly robust bacterial viability over a 49-day period. This substantiates the potential application of the tested materials as carriers for bacterial inoculum. The ability to maintain high viability over an extended period at room temperature presents an advantageous property for storing immobilized bacteria on a commercial scale. However, the required duration of viability for immobilized bacteria varies depending on the specific application. Applications demanding immediate bioremediation of contaminants necessitate short-term bacterial viability, whereas long-term applications, such as the gradual release of secondary metabolites with specific bioactivity, may require prolonged bacterial viability (Safari et al., 2020).

Our findings are consistent with previous studies, which have also demonstrated the potential of the four lignocellulosic materials employed in this research as carriers for bacterial inocula with diverse bioactivities, exhibiting considerable cell viability. For instance, in one study, rice husk powder was utilized as a carrier for a consortium of oil-degrading bacteria, successfully maintaining the viability of immobilized cells for a period of up to six months. Moreover, the immobilized bacterial consortium exhibited significantly higher oil degradation efficiency compared to the free-living bacterial inoculum used for comparison (Nunal et al., 2014). In wastewater treatment, rice husk is often used as a sole carbon source and also as a biofilm carrier for the biological denitrification process (Shao et al., 2009; Yu et al., 2019). Further, plant growth-promoting *Bacillus circulans* immobilized in rice husk was reported to maintain 2.381×10^5 CFU/g cell viability on the 63rd day after incubation (Gunjal et al., 2012). *Rhodococcus* sp. and *Pseudomonas* sp.

immobilized in powdered corn husk showed their application in the bioremediation of hydrocarbon-contaminated soils (Rivelli et al., 2013). Moreover, sugarcane bagasse used to immobilize a strain of *Bacillus pumilus* was capable of degrading a herbicidal compound, mesotrione (Liu et al., 2015). This formulation gave over 95% degradation of mesothrione within 4 days. Further, they revealed that after immobilization, bacterial cells were strongly absorbed and fully dispersed on the bagasse surface. Apart from its wide use as a carrier of bacteria in soil applications, sodium alginate has been evaluated for carrying bacteria for the bioremediation of contaminated water. For instance, alginate encapsulated-*Pseudomonas aeruginosa* and *Bacillus* sp. were able to retain the degradation capacity of a mixture of hydrocarbons for up to 30 days in artificial seawater contaminated with crude oil (Rahman et al., 2006). Further, a strain of *Streptomyces* sp. encapsulated in alginate showed 90% of removal of xylene from contaminated water (Chikhi et al., 2016). Interestingly, the encapsulation of bacteria into alginate was reported to have more efficient bioactivity than free bacteria cells (Patil et al., 2004).

Table 1: Viability of immobilized bacteria in carrier materials over a period of 49 days storage at room temperature

Carrier material	Colony forming units (CFU)/g ± SD ×10^7							
	Days after bacteria immobilization							
	1	7	14	21	28	35	42	49
Sugarcane bagasse	181.5±7.8	231.5±14.8	91.5±26.1	113.0±15.6	118.0±11.3	50.0±14.1	20.8±0.99	9.7±0.6
Rice husk	90.5±0.71	79.5±0.7	29.5±0.71	33.0±0.5	13.6±0.3	5.3±0.1	10.4±0.57	10.3±0.4
Corn husk	65.5±12.0	87.0±14.1	138.0±15.6	98.5±19.1	153.5±20.5	97.5±13.4	92.5±12.0	34.5±27.6
Rice straw	61.5±6.4	65.5±2.1	75.5±0.7	126.0±15.6	212.0±7.1	225.0±7.1	180.1±0.1	123.0±14.1
Sodium alginate	2.7±0.0.2	2.5±0.01	2.3±0.7	2.4±0.3	1.2±0.03	1.4±0.2	1.4±0.04	1.7±0.1

3.3. Impact of carrier materials on the water quality

Water quality stands as one of the most crucial determinants of the survival of aquatic biota and the ultimate success or failure of any aquaculture operation. It hinges on a myriad of physicochemical parameters, offering valuable insights into the availability of resources necessary to sustain life within the ecosystem. Thus, the assessment or monitoring of water quality becomes of paramount importance following the introduction of any foreign material into an aquatic environment (Thirupathaiah et al., 2012). In this study, we measured the physicochemical parameters of water after introducing sachets containing carrier materials into the water, aiming to ascertain the impact of these carriers on the physicochemical properties of the water 49 days after their introduction (Table 2).

To ensure the reliability of the data, the water used in this experiment was boiled tap water, as the disinfection process using chloride ions could be effectively eliminated by boiling (Zhang, 2013). The presence of chloride ions can adversely interfere with the accurate estimation of COD (Dobbs and Williams, 1963). This interference occurs because, in the absence of an adequate level of organic compounds in the reaction mixture, dichromate tends to oxidize chloride ions, resulting in a false positive COD reading. Therefore, the removal of chloride ions from water before COD estimation becomes an essential step (Kolb et al., 2017).

We observed significant (p<0.05) changes in the pH of the carrier material-introduced water at the end of the experiment compared to the initial pH (Table 2). According to the Tukey pairwise comparison (Abdi and Williams, 2010), the pH of water containing sodium alginate and rice husk clustered with that of the control water sample (Table A1 in Appendix A). This clustering revealed the variation of pH in water containing sodium alginate and rice husk grouped together with the pH of the control water sample without having a significant difference. Maintenance of stable pH is a property of a suitable carrier material to be introduced into the natural environment. Acidification of water can be considered unfavorable due to the depletion of water quality (APHA, 2005). Our results showed that sodium alginate, sugarcane bagasse, and rice husk were able to maintain near neutral pH in water during the experiment whereas, pH was found reduced with corn husk and rice straw. The decomposition of products of rice straw that were released into the water such as organic acids like citric, oxalic, formic, and maleic acids could be responsible for the lowered pH (Kumari et al., 2008).

Aquatic organisms, both plants, and animals, are known to be highly sensitive to even minor fluctuations in water pH (Lindholm et al., 2021). The optimal pH range that supports aquatic life typically falls between 6.0 to 9.0 (Thurston et al., 1981). Moderately acidic waters with a pH of 4.0 have been shown to have adverse effects, including a reduction in the number of hatched fish eggs, irritation to the gills of fish and aquatic insects, as well as membrane damage (Omer, 2019). Water with extremely low or high pH levels can prove fatal to a wide range of aquatic organisms, and pH values below 4 or above 10 have been found to be lethal for most fish species (Omer, 2019). Amphibians are particularly vulnerable to low pH levels, as their skin becomes more susceptible to the harmful effects of contaminants (Cole et al., 1999). In contrast, highly acidic waters facilitate the dissolution of heavy metals such as cadmium, lead, and chromium, rendering these metals much more toxic once dissolved in the water (Jaishankar et al., 2014).

Furthermore, variations in pH can lead to changes in the chemical structure of compounds present in water, thus affecting aquatic plants and animals. For example, ammonia is relatively harmless to fish in neutral or acidic water. However, as the water becomes more alkaline, ammonia becomes increasingly poisonous to the same organisms (DeZuane, 1997). Therefore, the reduction in the pH of water resulting from the addition of rice straw and corn husk carrier matrices may have a detrimental impact on aquatic biota.

Table 2: Physicochemical properties of water after 49 days of introducing carrier materials

	Water	Sodium alginate	Sugarcane bagasse	Rice husk	Corn husk	Rice straw
pH	7.46±0.03	7.68±0.00	6.82±0.02	7.54±0.01	5.41±0.02	5.69±0.01
TDS/(ppm)	82.00±0.00	114.00±0.00	101±0.00	103.00±0.00	104.00±0.00	119.00±0.00
EC/(μS/cm)	165.00±0.0	228.00±0.00	202.00±0.00	206.00±0.00	207.67±0.58	238.33±0.58
Salinity/(PSU)	0.08±0.00	0.10±0.00	0.09±0.00	0.10±0.00	0.10±0.00	0.11±0.00
Turbidity/(FNU)	2.83±0.57	2.50±0.30	80.13±3.35	6.43±0.15	69.20±1.40	62.90±3.72
Nitrate/(ppm)	0.57±0.02	0.63±0.01	0.56±0.02	0.49±0.02	0.31±0.02	0.30±0.03
DO/(ppm)	8.14±0.06	7.58±0.06	4.94±0.02	7.35±0.05	4.54±0.04	5.58±0.06
COD/(ppm)	1.00±0.00	33.67±5.51	480.33±10.97	105.00±3.61	739.00±51.12	663.00±14.73
COD/(ppm) with bacteria immobilized materials		65.27±2.57	213.67±14.15	77.67±1.53	164.00±1.00	258.77±3.62

Dissolved inorganic and organic substances in water play a crucial role in determining the conductivity, salinity, and total dissolved solids (TDS) of the water. In this experimental study, a consistent trend was observed in the alteration of conductivity, salinity, and TDS over time (Table 2). Conductivity represents a property associated with the ionic content of a sample, which, in turn, is influenced by the presence of dissolved (ionizable) solids (Barceló, 1993). Notably, the conductivity of all treatments and the control water gradually increased over time ($p<0.05$). Conductivity serves as an indirect measure of the saltiness of the water. Freshwater fish and other organisms have limited tolerance to significant increases in water salinity, as they are adapted to thrive within specific salinity ranges. Beyond this range, their survival becomes jeopardized (Grzesiuk and Mikulski, 2006).

The observed increase in conductivity of the water samples in this experiment might be attributed to the accumulation of inorganic ions resulting from the decomposition of organic matter within the carrier materials (Wang et al., 2017).

The determination of a water sample's salinity status relies on its electrical conductivity. The trend in the salinity of water samples introduced with carrier materials and the control group followed a similar pattern over time, aligning with their respective conductivity values. Salinity is quantified using "practical salinity units" (PSU), a unit of measurement that defines salinity based on the conductivity ratio of a sample to that of a solution containing 32.4356g of KCl at 15°C in a 1kg solution, as per the definition provided by the International Association for the Physical Sciences of the Ocean (IAPSO).

Throughout the experimental duration, all treatments and the control group exhibited a gradual increase in salinity ($p<0.05$). However, it is crucial to note that the salinity levels remained well below the threshold that could be harmful to phytoplankton and aquatic plants (Bertrand et al., 2017). TDS in water comprises inorganic salts such as chloride, calcium, magnesium, potassium, sodium, bicarbonates, sulfates, and dissolved organic matter. We posit that the decomposition of organic matter over time may have contributed to the increase in TDS observed in both the treatments and the control group. At the end of the 49-day period, the TDS levels in all treatments were approximately similar but slightly higher than those in the control group. It is important to note that high turbidity can have adverse effects on aquatic life by hindering light penetration, which is essential for photosynthesis. This, in turn, reduces the levels of dissolved oxygen and can lead to fish mortality.

Nitrate, as a major nutrient, significantly contributes to the productivity of an aquatic ecosystem. Excessive nitrate levels often result in the formation of dense phytoplankton biomass. Nitrate is formed through the nitrification process of organic matter, driven by aerobic bacteria. Any nitrate not assimilated by aquatic plants is primarily denitrified in anaerobic sediments, subsequently being released into the atmosphere (Ganesh et al., 2020). The lowest nitrate-N levels were measured from corn husk and rice straw-treated water. This result is in agreement with a previous study, which showed enhanced nitrogen removal from wastewater by increasing denitrification when treated with rice straw (Yang et al., 2015). The desired nitrate concentration favorable for plant and phytoplankton falls within the range of 0.2 to 10 mg/L (Sayyad, 2020). Our results showed that the effect of carrier materials on the nitrate concentration of water was negligible (Table 2).

Dissolves oxygen (DO) is another important parameter that critically determines the survival of aquatic life and reflects the physical and biological processes prevailing in that water (Devi et al., 2017). The desired concentration of DO range in water ranges from 5-15ppm for aquaculture practices. The DO of all treatments was in the favorable range throughout the experiment and also, the addition of carrier materials to the water appeared to have less effect on the DO (Table 2). According to the Tukey pairwise comparison, the DO of the control water sample, sodium alginate, and rice husk grouped together without any significant difference between these three tested samples (Table A1 in Appendix A). But for the corn husk, rice straw and sugarcane bagasse mean DO level was lower than 5ppm.

The chemical oxygen demand (COD) is a parameter that measures all organics *viz.*

biodegradable and non-biodegradable substances in a sample. The COD measures the amount of oxygen equivalent to the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant like dichromate (Geerdink et al., 2017). According to the results, the COD of the control water sample reduced over time from 17 ppm to 1 ppm (Table 2). Nonetheless, the COD of water-containing carrier materials exhibited an increasing trend over time. In comparison to the other carrier materials, the COD levels in water samples containing sodium alginate and rice husk were notably lower and demonstrated a clustering pattern with that of the control water in the Tukey pairwise comparison, with no statistically significant difference observed (Table A2 in Appendix A). A previous study also reported a low release of COD in rice husk treated- wastewater compared to rice straw- and corn husk-treated wastewater (Yang et al., 2015). Generally, the COD of water increases with increasing concentrations of organic matter and inorganic matter (Boyd, 1990). Given that all materials used in this experiment are readily biodegradable, albeit with varying degrees of efficiency, the release of organic and inorganic matter during the biodegradation process might have contributed to the observed fluctuations and differences in the COD levels of water-containing carrier materials. The variations in COD can also be attributed to the diverse compositions of the materials employed. Notably, the raw materials exhibit different lignin percentages, with approximately 13.4% in sugarcane bagasse, 20% in rice husk, 5.4% in rice straw, and 8.2% in corn husk (Wartelle and Marshall, 2006). In this experimental study, we anticipated a reduction in the lignin content of all materials compared to their raw state, as they were subjected to an alkaline pre-treatment aimed at lignin removal. However, there was no definitive indication or measurement in the pre-treatment process to ascertain the extent of lignin removal. Consequently, it is plausible that a certain amount of lignin might have persisted in the materials after the treatment, potentially contributing to the observed increase in COD levels over time.

When materials containing immobilized bacteria were introduced, the COD of water containing all carrier materials except sodium alginate was increased (Table 2). This may be due to the bacterial consumption of lignocellulosic and other organic compounds as their carbon source (Boer et al., 2005). It is known that bacteria are capable of utilizing cellulose and hemicellulose when cellulose was the sole carbon source (López-Mondéjar et al., 2016). The lowest COD was obtained for sodium alginate and rice husk. However, in contrast to our results, a previous study reported an 87% decrease in COD when *Bacillus*-immobilized rice husk was used in treating wastewater (Kennedy and Sekaran, 2004).

Upon careful consideration of the comprehensive results from the experiment, it became evident that the physicochemical properties of water containing the introduced carrier materials, as well as the control water, underwent changes over time ($p<0.05$). However, it is worth noting that the pH of the control water remained unchanged throughout the experimental duration. In order to identify suitable carrier materials that would have minimal impact on the water quality of the receiving water body, we conducted a statistical comparison between the changes in water quality parameters following the introduction of the tested materials and those observed in the control water. The analysis revealed that the mean values of all parameters in water containing sodium alginate were grouped together with the control water sample, and no significant differences were observed in pH, COD, DO, nitrate, TDS, and turbidity at the end of the 7th week (Tables A1, A2, A3, and A4 in Appendix A). Consequently, it appears that the introduction of sodium alginate may not lead to substantial changes in the water quality of the receiving aquatic environment. Additionally, the water samples containing rice husk showed similarities with the control and sodium alginate-introduced water regarding their COD, DO, pH, nitrate-N concentration, and turbidity. A two-sample t-test was also conducted, revealing no statistically significant difference ($p>0.05$) between the water samples containing rice husk and those containing sodium alginate in terms of pH, EC, salinity, DO, nitrate-N concentration, and turbidity. In summary, these analyses demonstrated that the alteration in water quality upon the addition of rice husk was not significantly different from that observed with sodium alginate, which is a widely utilized bacterial carrier material in various environmental applications.

The sole matrix of rice husk has the potential to act as an adsorbent (Ahmaruzzaman and Gupta, 2011). It has been shown to adsorb heavy metal contaminants in wastewater such as lead (Abdel-Ghani et al., 2007), arsenic (Khalid et al., 1998), Fe, Mn, Zn, Cu, and Cd (Daifullah et al., 2003) in wastewater. Further, activated charcoal produced from rice husk has been identified with the potential to adsorb phosphorous from wastewater (Abdul and Aberuagba, 2005). Alginate is also considered to play a major role in water treatment acting as an adsorbent of heavy metal ions (Dechojarassri et al., 2018). For instance, calcium alginate beads can act as a green sorbent for the selective recovery of Cu (ii) in polluted water (Yang et al., 2019). Therefore, it appears that, when rice husk and sodium alginate are used as carriers of bacteria, those two materials alone may provide additional bioremediation potential in heavy metal-contaminated waters.

3.4. Comparison with ambient water quality standards

Water quality parameters of carrier material-introduced water were subjected to a comparative assessment against the guidelines for drinking water provided by the World Health Organization (WHO, 2011) and the Ambient Water Quality Standards outlined in the National Environmental Regulations of Sri Lanka No. 01 of 2019. The objective was to ascertain the suitability of water for various purposes following the application of the tested materials as carriers for bioinoculants (Table 3). Given that the majority of freshwater bodies in Sri Lanka serve multiple purposes, all six types of national ambient water quality standards were taken into consideration. It was observed that with the exception of COD, the other water quality parameters in sodium alginate- and rice husk-introduced water met the minimum standards set by the WHO for drinking water and the national standards for various purposes, including drinking and other uses. Consequently, sodium alginate and rice husk materials emerge as favorable options as carriers for immobilizing bioinoculants, such as bacteria, for the purpose of bioremediating polluted freshwater environments. However, owing to the observed increase in COD levels, we recommend the collection of carrier materials after the successful establishment of the introduced bioinoculum in the receiving water. This approach would serve to mitigate any potential impacts arising from the increase in COD and ensure the overall effectiveness of the bioremediation process.

Table 3. Comparison of quality parameters of water introduced with carrier materials with WHO and Ambient water quality standard by National Environmental Regulations, Sri Lanka

	WHO	Ambient water quality standards of National Environmental Regulations					
		A	B	C	D	E	F
EC µS/cm, max	400 SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	700 SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS
Turbidity NTU, max	5.0 SA	5.0 SA	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS
pH	7.0-8.5 SA, RH	6.0-8.5 SA, SCB, RH	6.0-9.0 SA, SCB, RH	6.0-8.5 SA, SCB, RH	6.0-9.0 SA, SCB, RH	6.0-8.5 SA, SCB, RH	5.5-9.0 SA, SCB, RH, CH, RS
DO at 25 °C mg/l, min	* SA, SCB, RH, CH, RS	6.0 SA, RH	5.0 SA, RH, RS	5.0 SA, RH, RS	4.0 SA, RH, RS	3.0 SA, RH, CH, RS	3.0 SA, RH, CH, RS
NO_3-N mg/l, max	50 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS	10 SA, SCB, RH, CH, RS
TDS/ppm, max	600 SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS	* SA, SCB, RH, CH, RS
COD mg/l, max	80 SA	10 -	10 -	15 -	30 -	* SA, SCB, RH, CH, RS	40 SA

CH: Corn husk; RH: rice husk; RS: rice straw; SA: sodium alginate; SCB: sugarcane bagasse; *: Value not defined; Max: Maximum permissible level; Min: Minimum required level; A: Drinking (with simple treatment); B: Recreation; C: Aquatic life; D: Drinking (with general treatment); E: Irrigation and agriculture; F: Minimum quality than A, B, C, D, E

4. Conclusion and recommendations

Alkaline pretreatment applied to all tested green carrier materials, namely corn husk, rice husk, rice straw, and sugarcane bagasse, resulted in a significant enhancement of bacteria immobilization. Moreover, the immobilized bacteria demonstrated robust viability in these four carrier materials and sodium alginate, maintaining approximately 107 CFU per gram of material after 49 days of storage at room temperature. The substantial immobilization and prolonged viability of bacteria in these carrier materials indicate their potential as efficient carriers of bacterial inocula. Upon introducing the carrier materials to the water, there was no significant increase in the availability of nitrate-N, a major nutrient that influences the trophic status of an aquatic ecosystem. However, the introduction of carrier materials did lead to alterations in other physicochemical properties of the water, including changes in pH, EC, salinity, DO, turbidity, TDS, and COD. To evaluate the impact of the carrier materials on water quality, these parameters were compared with the ambient water quality standards, specifically the WHO standards for drinking water and the Sri Lankan ambient water quality regulations for drinking and other purposes. The introduction of rice husk and sodium alginate as carrier materials did not have a significant impact on the quality parameters used in the determination of WHO standards for drinking water and Sri Lankan ambient water quality regulations for drinking and other purposes. However, it is noteworthy that the COD of the receiving water increased over time and exceeded the maximum permissible levels defined in water quality standards. In conclusion, we emphasize the potential application of sodium alginate and alkali-treated rice husk as suitable carriers for delivering bacterial inocula to freshwater environments. To minimize the impact of carrier materials on the COD of water, we strongly recommend collecting the carrier materials after the establishment of the bioinoculum in the receiving water. This precautionary approach will ensure the effective bioremediation of polluted freshwater environments while safeguarding water quality standards.

Appendix A. Comparison of physicochemical properties of water

Tables A1 to A44 present the outcomes of Tukey pairwise comparisons for the average water quality parameters, namely pH, dissolved oxygen, chemical oxygen demand, nitrate-N, electrical conductivity, salinity, total dissolved solids, and turbidity. The assessments were conducted on water samples treated with different carrier materials, including sodium alginate (SA), alkali-treated rice husk (TRH), alkali-treated sugarcane bagasse (TSCB), alkali-treated rice straw (TRS), and alkali-treated corn husk (TCH). The primary objective was to investigate the performance of these materials in enhancing water quality. To establish meaningful comparisons, the results were compared with those obtained from a control water sample.

Table A1: Pairwise comparison of pH of water containing tested carrier materials (95% confidence)

Factor	N	Mean	Grouping			
SA pH	12	7.4683	A			
TRH pH	12	7.4092	A			
control pH	12	7.2217	A			
TSCB pH	12	6.800		B		
TRS pH	12	6.1983			C	
TCH pH	12	4.8883				D

This means that do not share a letter that is significantly different

Table A2: Pairwise comparison of dissolved oxygen (DO) of water containing tested carrier materials (95% confidence)

Factor	N	Mean	Grouping	
control DO	12	7.989	A	
SA DO	12	7.202	A	
TRH DO	12	6.125	A	B
TSCB DO	12	4.943		B
TRS DO	12	4.571		B
TCH DO	12	4.304		B

This means that do not share a letter that is significantly different

Table A3: Pairwise comparison of chemical oxygen demand (COD) of water containing tested carrier materials (95% confidence)

Factor	N	Mean	Grouping		
TCH COD	12	809.1	A		
TRS COD	12	487.9		B	
TSCB COD	12	395.7		B	
TRH COD	12	109.42			C
SA COD	12	55.17			C
control COD	12	5.42			C

This means that do not share a letter that is significantly different

Table A4: Pairwise comparison of nitrate-N (NIT), electrical conductivity (EC), salinity (Sal), total dissolved solids (TDS), and turbidity of water containing tested carrier materials (95% confidence)

Factor	N	Mean	Grouping		
Control NIT	12	0.6183	A		
TSCB NIT	12	0.5633	A		
TRH NIT	12	0.5550	A		
SA NIT	12	0.5450	A		
TRS NIT	12	0.3333		B	
TCH NIT	12	0.1850		B	
TRS EC	12	212.17	A		
SA EC	12	208.58	A	B	
TCH EC	12	206.83	A	B	
TRH EC	12	199.75	A	B	
TSCB EC	12	186.33		B	
Control EC	12	146.67			C
TRS Sal	12	0.10000	A		
TCH Sal	12	0.09750	A		
SA Sal	12	0.09667	A	B	
TRH Sal	12	0.09333	A	B	
TSCB Sal	12	0.08500		B	
Control Sal	12	0.06750			C
TRS TDS	12	106.08	A		
SA TDS	12	104.17	A	B	
TCH TDS	12	103.500	A	B	
TRHTDS	12	99.83	A	B	
TSCB TDS	12	93.17		B	
Control TDS	12	73.25			C
TCH TURBIDITY	12	54.46	A		
TRS TURBIDITY	12	45.89	A		
TSCB TURBIDITY	12	39.89	A		
TRH TURBIDITY	12	6.675		B	
SA TURBIDITY	12	2.858		B	
Control TURBIDITY	12	2.008		B	

This means that do not share a letter that is significantly different

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Compliance with ethical standards

Conflict of interest

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