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Areca nut (*Areca catechu*) husks and Luffa (*Luffa cylindrica)* sponge as microbial immobilization matrices for efficient phenol degradation

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ABSTRACT

Immobilization of microorganisms is a widely adopted strategy for the efficient degradation of hazardous organic compounds like phenol. Microorganisms can be immobilized in synthetic or natural matrices. In this work dried areca nut (*Areca catechu*) husks and luffa (*Luffa cylindrica*) sponge fibers were used as alternative and inexpensive natural matrices for microbial cell immobilization. The potential of these immobilization systems for the effective bioremediation of phenolic wastewater was explored. A bacterial consortium was isolated by enriching a sludge sample from a petroleum refinery in high phenol concentrations. The mixed bacterial culture was capable of degrading 1000 mg L^{-1} of phenol in suspension cultures. The bacterial consortium was immobilized on the lignocellulosic matrices. Phenol degradation studies were performed in batches to optimize the physicochemical parameters. Optimum pH and temperature for phenol degradation was found to be 8.0 and 37 °C. At an optimum pH and temperature, the areca nut husk and luffa sponge systems immobilized with the mixed culture could degrade 1000 mg L^{-1} phenol in 28 h and 30 h respectively. The highest experimental degradation rates in areca nut husk and luffa sponge systems were 0.37 h^{-1} and 0.21 h^{-1} respectively at 200 mg L^{-1} phenol. Degradation kinetic studies were carried out using several inhibition models. Further studies revealed that both matrices with immobilized microbes could be reused for several successive batch degradation experiments and stored at 4 °C for several weeks without any noticeable loss in degradation efficiency.

1. Introduction

Phenols or phenolics are a class of organic chemical compounds well known for their toxic effects on microbes, aquatic flora and fauna, animals and humans even at low concentrations. The simplest of them is phenol, which is the most sought-after pollutant due to its recalcitrant and ubiquitous nature. Phenolic compounds are very frequently found in run-offs and effluents of various industries such as petrochemicals, coal conversion, coking plant, leather, textiles, pharmaceutical, olive oil, paint, polycarbonate resin, ink, paper, perfume etc. [1]. Phenol has been categorized in the list of significant contaminants by the US Environmental Protection Agency (USEPA) due to its high solubility in water sources and toxicity [2]. The permissible limits of phenol in potable waters has been set to 1 μg L^{-1} by the World Health Organization (WHO) [3]. Hence, removal of phenol from wastewater is essential before its release into the environment.

Over the years researchers have shown that bioremediation is a cost-effective and eco-friendly alternative to the traditional physico-chemical methods used for phenol removal [4–7]. Although sustainable, the inhibitory effect of phenol at high concentrations pose a serious challenge for wastewater remediation by microorganisms [8]. Hence, several strategies have been explored to overcome this problem. Most common approaches include use of genetically modified organisms, acclimatization of organisms to lethal phenol concentrations, and immobilization of organisms [9]. Immobilization of microbial cells offer several benefits over other approaches for treatment of phenolic wastewaters: (1) fortifies microbes against the toxic effects of phenol and harsh environments, (2) helps reach a high microbial concentration hence achieving a high contaminant degradation rate, (3) helps easy recovery and re-use of microbial cells for repeated degradation of target pollutants and (4) helps microbial cells stay viable during storage for extended durations with little or no loss of degradation capacity hence ensuring stable long-term operation [5,10,11]. Immobilization by entrapment of microbial cells in matrices such as calcium-alginate beads [8,12] or a more mechanically stable PVA (poly vinyl alcohol) [4,6,13] were reported earlier. Entrapment of whole cells using chitosan beads [10] or hybrid systems involving alginate-chitosan, PVA-alginate and glycerol-alginate [9] were also implemented. Immobilization studies

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Table 1
Comparison of reported immobilization matrices for phenol degradation.

Sl. No.	Immobilization matrix	Pollutant/ concentration	Organism/s used	Experimental degradation rate, q (h^{-1})	Reference
1	Microfiltration membrane capsule	Phenol@100 mg L^{-1}	*Pseudomonas putida* F1	1.05	[32]
2	PVA Gel	Phenol@800 mg L^{-1}	*Acinetobacter*sp. XA05 and *Sphingomonas* sp. FG03 (1:1 ratio)	1.2	[13]
3	PVA Gel	Phenol@500 mg L^{-1}	*Acinetobacter* sp. strain PD12	1.249	[6]
4	Calcium Alginate	Phenol@500 mg L^{-1}	*Bacillus cereus* MTCC9817	0.26	[7]
5	Calcium Alginate	Phenol@100 mg L^{-1}	*Bacillus cereus* MTCC 9818	0.15	[7]
6	Areca nut husk	Phenol@200 mg L^{-1}	Mixed culture	0.37	This work
7	Luffa fibres	Phenol@200 mg L^{-1}	Mixed culture	0.21	This work

were carried out using supports such as polyurethane foam (PUF) [14], Celite R-635 [15] and Liapor clay beads [16]. A major drawback of systems such as calcium-alginate is its instability against phosphates and disruption of gel particles due to CO_2 evolution [17].

In recent years, the pursuit for finding a natural, renewable, non-toxic and biodegradable immobilization matrix has brought lignocellulosic biomass to limelight. Microbial cells immobilize onto lignocellulosic biomass via physical adsorption [18]. Owing to its abundance on planet Earth, a variety of lignocellulosic supports such as bagasse, sawdust, wood shavings/chips, rice husk, straw and dry coconut husk were tested for fermentation and wastewater treatment studies [5,17,18]. Table 1 describes various reported immobilization matrices used for phenol degradation. In the current study, we have used dried areca nut husks and dry mature luffa vegetable fibers as potential candidates to immobilize bacteria for phenol degradation.

Areca catechu (Family: Arecaceae) is a species of palm, which grows lavishly in Asian countries. The fruit is known as areca nut or betel nut. India is the largest producer of areca nut in the world [19]. The fruit is protected by a fibrous husk, which is separated before consumption of the areca nut. This husk is discarded and is considered as agricultural waste [19]. Owing to the considerable amount of sugars (21%) in these husks, they have been used as low-cost substrates for citric acid production by solid state fermentation [20]. On the other hand, *Luffa cylindrica* (Family: Cucurbitaceae) is a vegetable crop that grows abundantly in tropical and sub-tropical climates of African and Asian countries. The fruit, although edible at a younger stage, becomes very fibrous once ripened. The mature fruit is dried until the fleshy part fades away leaving a skeleton of randomly interconnected network of highly fibrous matrix. The dried fruit is widely used as bath scrubber and for manufacturing bath mats, table and door mats [21]. These fibers are highly porous and are used as a potential support for immobilization of microbial cells for ethanol production [21,22]. The use of luffa sponge for immobilization of algal and fungal cells for removal of heavy metals was also reported [23]. However, to our knowledge, there are no reports on bacterial immobilization on areca nut husk and luffa fibers for treatment of phenolic wastewater.

Therefore, we have explored the potential of these two lignocellulosic biomasses as carrier matrices for bacterial immobilization and their use for treatment of synthetic phenolic wastewater in this study. A mixed bacterial culture, which was previously acclimatized to high phenol concentrations, was immobilized onto these two biomasses. The effect of factors such as pH of the growth medium, temperature of incubation and initial phenol concentration were studied and optimized. The experimental degradation data were fitted to various established substrate inhibition models via MATLAB R2018A and various bio-kinetic parameters were estimated.

2. Materials and methods

2.1. Chemicals and raw materials

All the analytical grade chemical components and phenol crystals were procured from HiMedia® and MERCK India. Areca nut husks and luffa sponges were obtained from the local market in Guwahati, Assam, India.

2.2. Microorganisms and culture condition

A microbial consortium was isolated in our earlier study [24] by enriching a sludge sample acquired from a petroleum refinery in high phenol concentrations. The mixed microbial culture could utilize phenol up to a concentration of 1000 mg L^{-1}. The enrichment culture contained three culturable strains of bacteria. These isolates were identified as Brevibacterium sp. DBK1 (NCBI GenBank Accession no: KP231222), Stenotrophomonas acidaminiphila DBK (NCBI GenBank Accession no: KC992293) and Brucella sp. DBK2 (NCBI GenBank Accession no: KP231223) by 16S rRNA sequencing. The mixed culture was maintained and cultivated in sterilized MSM (mineral salt medium), the chemical composition of which has been described in our previous report [24]. Sub-culturing was done every fifteen days in an incubator shaker at 37 °C and 120 rpm (Incubator Model: ZHICHENG Instruments ZHWY-2112B) with 1000 mg L^{-1} of phenol as a sole energy and carbon source. The mixed culture, at its late log phase was stored at 4 °C.

2.3. Preparation of the lignocellulosic matrices and immobilization of the mixed culture

Areca nut husks were washed with water and dehydrated in a hot air oven at 85 °C to remove any moisture, as these are prone to fungal contamination. The outside skin or the exocarp of the areca nut husks were removed and only the fibrous part or mesocarp was retained. The luffa sponges were also washed with water and dried at 85 °C to remove moisture.

The dried areca nut husks and mature luffa sponges were then chopped into pieces of 5 mm–6 mm approximately. The chopped biomasses were washed several times with deionized water to remove pigments imparted by the particles. These were dried again at 85 °C. The untreated biomasses were autoclaved, dried and stored. These were later used as matrices for microbial immobilization. Immobilization of microbial cells onto the dried biomass was performed *in situ* in conical flasks by a semi-solid state approach, where cells were immobilized by natural adsorption [5,17].

20 g of sterile and dried areca nut husk was mixed with 200 mL of fresh bacterial culture. The bacterial culture was maintained in 1000 mg L^{-1} phenol for achieving higher bacterial biomass densities. The concentration of the bacterial culture was recorded as 1.09 (OD_{600}) which was equivalent to 0.944 g L^{-1} dry cell weight (DCW) (1.0 OD equivalent to 0.866 g L^{-1} of dry cell biomass, $R^2 = 0.97$, data not

shown here). This was allowed to stand in a static incubator at 37 °C. After 24 h, the flasks were augmented with 50 mL of fresh media substituted with 200 mg L^{-1} phenol and allowed to stand overnight. Thereafter, the entire media was replaced with 200 mL of fresh mineral media containing another 200 mg L^{-1} phenol. This was allowed to stand for another 24 h in a static incubator at 37 °C. This method of immobilization was adopted from Basak et al. [5] with slight modifications.

Similarly, immobilization of bacterial culture was also carried out for luffa sponges. Since the density of luffa sponge is rather low when compared to areca nut husks, only 6 g of dry luffa sponges were used for immobilization of bacterial culture. The entire immobilization process was achieved in 500 mL conical flasks containing 200 mL of bacterial culture.

2.4. Optimization of physical parameters responsible for growth and substrate utilization (batch studies)

2.4.1. Effect of initial pH of the culture media

Batch experiments were performed to ascertain the impact of initial pH of the growth medium on the bioremediation of phenol. A wide range of pH levels were considered for the experiment (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). The selection of pH values were made based on our previous study [24] on phenol degradation using free cells of the mixed bacterial culture. The initial pollutant concentration in the culture medium was maintained at 200 mg L^{-1}. Experiments were performed in 250 mL conical flasks containing 100 mL of culture media. 15 g of wet areca nut husk and 6 g of wet luffa sponge fibers (with immobilized microorganisms) were used as inoculum for the experiments. All the experiments were carried out at 37 °C in an incubator shaker at 120 rpm. Phenol degradation was monitored at regular intervals. Degradation data was used to calculate the degradation rate of phenol, which helped determine the optimal pH required for substrate utilization.

2.4.2. Effect of incubation temperature

Experiments were also performed to investigate the impact of temperature of incubation on the bioremediation of phenol. A predetermined optimum pH, calculated from the previous set of experiments, was used for the study. 200 mg L^{-1} of phenol was used as a solitary source of carbon for the experiments. Studies were performed at incubation temperatures of 25 °C, 30 °C, 37 °C and 45 °C in 250 mL conical flasks containing 100 mL of culture media in a shaker incubator at 120 rpm. The inoculum volume was maintained at 15 g for wet areca nut husks and 6 g for wet luffa sponge fibers. Consumption of phenol by the immobilized microbes was monitored at regular intervals for calculation of phenol degradation rate and successive ascertainment of the optimum incubation temperature.

2.4.3. Effect of initial phenol concentrations

At an optimum pH and temperature determined from the previous set of experiments conducted in Sections 2.4.1 and 2.4.2 respectively, studies were also conducted to ascertain the optimum phenol concentration for rapid substrate utilization. Various increasing concentrations of phenol (200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}) were used as the sole source of carbon and energy for this study. Inoculum load was maintained at 15 g for wet areca nut husk and 6 g for wet luffa sponge fibers. Experiments were performed in 250 mL conical flasks containing 100 mL of mineral media in a shaker incubator at 120 rpm. Residual concentrations of phenol were estimated by withdrawing samples at fixed intervals. Phenol degradation data was used for calculation of degradation rate, q (h^{-1}) and analysis of degradation kinetics. Degradation kinetic study is discussed in a later section.

The optimum value of environmental parameters was determined based on the value of degradation rate, q (h^{-1}). All experiments discussed above were conducted in triplicates.

2.5. Analytical methods

2.5.1. Analysis of residual phenol

Nylon membrane filter papers (0.22 µm pore size, Pall Corporation) were used to filter samples. Residual phenol concentration in the filtrates were determined via High Performance Liquid Chromatography (Model: Agilent Technologies 1220 Infinity LC) fitted with a Variable Wavelength Detector (VWD) and a reverse phase column (Agilent ZORBAX Eclipse XDB-C18, particle size 5 µm, dimensions 3.0 x 150 mm) after appropriate dilutions. A mobile phase of water (60%): acetonitrile (40%) was used and a flow rate of 1.0 mL min^{-1} was maintained at room temperature. Residual phenol was detected by injecting aliquots of 20 µL at a wavelength of 280 nm.

2.5.2. Electron microscopic studies

The lignocellulosic matrices with and without the immobilized mixed bacterial consortium was observed and studied via FESEM (Field emission scanning electron microscopy). 5 mm pieces of areca nut husk and luffa sponges were collected from the immobilization flasks and fixed by treatment with 3.5% glutaraldehyde for 6 h [17]. These were further washed with sterile crystal-free neutral phosphate buffer and dehydrated by treatment with increasing ethanol gradation (50%, 70%, 90%, 95% and 100%). The samples were finally dried in a vacuum desiccator overnight. The dried samples were directly placed on a double-sided carbon tape on a FESEM stub and coated with gold film. The morphology of the bacterial community immobilized on the lignocellulosic matrices were witnessed and photographed at a magnification of 0.5–10 KX via Zeiss FESEM (model: Sigma). The oven-dried lignocellulosic matrices without the immobilized bacterial culture were directly viewed under FESEM without any kind of pre-treatment.

2.6. Degradation kinetic studies and comparison of inhibition kinetic models

Experimental degradation data (obtained from batch studies described in Section 2.4.3) was fitted to several available kinetic models to elucidate the kinetics of phenol degradation by the immobilized microorganisms. Various established substrate inhibition kinetic models viz., Haldane model [25], Yano model [26], Aiba model [27], Edward model [28] and Webb model [29] (Table 2) were considered in this study. Degradation rate of phenol, q (h^{-1}), was ascertained from the gradient of plot of the negative logarithm of S/S_o vs. time (t) [7] for various initial substrate concentrations (S_o). The plot of experimental degradation rates (q) versus values of various corresponding initial phenol concentrations (S_o) were fitted to the different substrate inhibition models. Different bio-kinetic parameters such as, q_{max} (maximum degradation rate, h^{-1}), K_I (inhibition constant, mg L^{-1}), and K_S (affinity constant, mg L^{-1}) were calculated for the inhibition models using nonlinear regression analysis in MATLAB R2017b.

2.7. Storage and reusability studies of the mixed culture immobilized on the lignocellulosic biomass

The effect of storage on the stability and biodegradation potential of the microbes immobilized on the lignocellulosic biomass was studied for a period 0–6 weeks. After the immobilization of the mixed bacterial culture on the lignocellulosic biomass (as described in Section 2.3), a biodegradation experiment was conducted for a phenol concentration of 200 mg L^{-1}. Residual phenol concentrations were measured at regular intervals and time taken for complete phenol degradation was noted. In order to maintain similar inoculum quality for the entire course of storage stability experiments, abundant immobilized material was prepared for carrying out biodegradation studies in triplicates for eight batches. The immobilized cells were then stored at 4 °C for conducting the storage stability experiments. Biodegradation batch

Table 2
Model fitting and calculation of bio-kinetic parameters.

Immobilization matrix	Model	q_{max} (h^{-1})	K_S (mg L^{-1})	K_I (mg L^{-1})	K (mg L^{-1})	R^2
Areca nut husk	Haldane model: $q = \frac{q_{max}S}{K_S + S + \left(\frac{S^2}{K_I}\right)}$ [25]	11.1632	1805.71	10	–	0.9218
	Aiba model: $q = \frac{q_{max}S}{K_S + S} exp\left(\frac{-S}{K_I}\right)$ [27]	0.6053	20.53	515	–	0.9706
	Edward model: $q = q_{max}S\left[exp\left(\frac{-S}{K_I}\right) - exp\left(\frac{-S}{K_S}\right)\right]$ [28]	**0.0042**	**3.58**	**231**	–	**0.9858**
	Yano model: $q = \frac{q_{max}S}{K_S + S + (S^2/K_I)[1 + S/K]}$ [26]	0.7446	105.02	893	173.17	0.9626
	Webb model: $q = \frac{q_{max}S[1 + S/K]}{S + K_S + (S^2/K_I)}$ [29]	162.1371	27031.92	1	22632.45	0.9183
Luffa fibers	Haldane model	5.2247	2298.7	16	–	0.9681
	Aiba model	**0.3413**	**39.5**	**663**	–	**0.9903**
	Edward model	0.002	5.6	273	–	0.9815
	Yano model	0.3958	104.78	832	368.32	0.9877
	Webb model	4.1883	1224.26	13	1436.41	0.9233

experiments were conducted in triplicates, every week (for up to 6 weeks), to check for the decline (if any) in biodegradation potential of the immobilized mixed microorganisms. Before commencing the batch experiments, the lignocellulosic biomass was allowed to reach the room temperature.

Reusability of the immobilized microorganisms was also studied by conducting several successive batch degradation experiments. For these studies, the lignocellulosic biomass immobilized with the mixed microbial culture was retrieved from the first batch of experiments. These were then washed with sterile distilled water. Subsequently, the retrieved biomass was subjected to repeated batch biodegradation experiments for up to 15 batches. After each batch, the biomass was retrieved and washed with sterile distilled water. Residual phenol concentration was monitored at regular intervals and time taken for complete degradation was noted for all the batches.

All the studies were conducted in triplicates with a phenol concentration of 200 mg L^{-1} in 250 mL conical flasks. A uniform pH and temperature of 8.0 and 37 °C respectively was maintained in the shaker incubator at 120 rpm for all storage and reusability experiments.

2.8. Composition analysis studies of the lignocellulosic biomass

Compositional analysis studies were carried out for the lignocellulosic biomass without immobilized microorganisms and with immobilized microorganisms after 15 consecutive batch experiments to determine the degradation of the structural components (if any) of the lignocellulosic biomass. Hemicellulose was determined by a method described by Goering and Van Soest [30] which involved estimating neutral detergent fiber (NDF) and acid detergent fiber (ADF) separately. Hemicellulose content can be determined by calculating the difference between NDF and ADF. Holocellulose was measured by treatment with sodium chlorite [31]. Lignin was estimated by TAPPI standard protocol (Technical association of the pulp and paper industry).

3. Results and discussion

3.1. Lignocellulosic matrices, immobilization of the mixed culture and electron microscopic imagery

Usually, microorganisms are immobilized onto solid supports by recirculating a concentrated microbial culture suspension through a reactor where the lignocellulosic materials act as solid supports. One major drawback of this process is cell desorption, which restricts operational stability [17]. In this study, a semi-solid-state approach was adopted to achieve maximum cell immobilization and enhanced stability. Adding fresh media with phenol after 24 h and 48 h (as discussed in Section 2.3.) resulted in enhanced cell growth, causing microbial cells to be immobilized thoroughly on the supports [17]. Scanning electron microscopy (FESEM) was performed to visualize the lignocellulosic matrices with and without immobilization of the mixed bacterial culture. Fig. 1a and b portrays the surface architecture of the areca nut husks and luffa sponge fibers respectively without immobilized microorganisms. It can be easily visualized that the surface of the areca nut husks is a lot rougher and porous in texture when compared to the luffa sponge fibers. Fig. 1(c and d) depicts the immobilization of the microbial cells on the areca nut husks. Copious amounts of bacterial cells can be seen immobilized on the surface of the areca nut husks. The surface architecture of the husks appears to be smooth (in contrast to the surface without microorganisms), which is possibly due to formation of biofilms. Fig. 1(e and f) illustrates the immobilization of microbial cells on the surface of the luffa sponge fibers. It can be observed that, when compared with areca nut husk fibers, the intensity of immobilized bacterial cells on the luffa sponges has declined. This impairment in immobilization intensity can be attributed to the fact that the surface of the luffa sponge fibers is smoother compared to the areca nut husks. The rougher surface of the areca nut husks might be responsible for enhanced cell adsorption. The bacterial cells were adsorbed on the lignocellulosic biomass naturally, which can be ascribed to covalent bonding or electrostatic forces between the surface of the lignocellulosic biomass and the bacterial cells [5].

3.2. Effect of initial pH on the degradation of phenol

pH plays a vital role in utilization and degradation of phenolic compounds by microorganisms. Maintaining an optimum pH of the culture media activates enzymes that are responsible for degradation of phenol. Extreme changes in pH renders these enzymes non-functional causing denaturation and loss of catalytic activity [24]. Denaturation of enzymes impairs metabolic activity of the microorganism eventually leading to rapid obliteration of bacterial biomass. Hence, an optimum initial pH should be imperatively maintained for efficient phenol degradation.

Fig. 2a represents the phenol degradation curves at different pH values in case of areca nut husk immobilization system. It can be observed that phenol was completely degraded in 6 h for pH 7.0 and pH 8.0. Whereas, complete degradation of phenol was achieved in 8 h for pH 5.0 and pH 6.0. For higher pH values of 9.0 and 10.0, more than 99% of the pollutant was degraded in 10 h. Fig. 2b represents the phenol degradation percentage and degradation rates for 200 mg L^{-1} phenol at different pH values in areca nut husk immobilization system. The degradation rate reached a maximum value of 0.37 h^{-1} at pH 8.0. Although complete degradation of phenol was achieved at pH 7.0, the

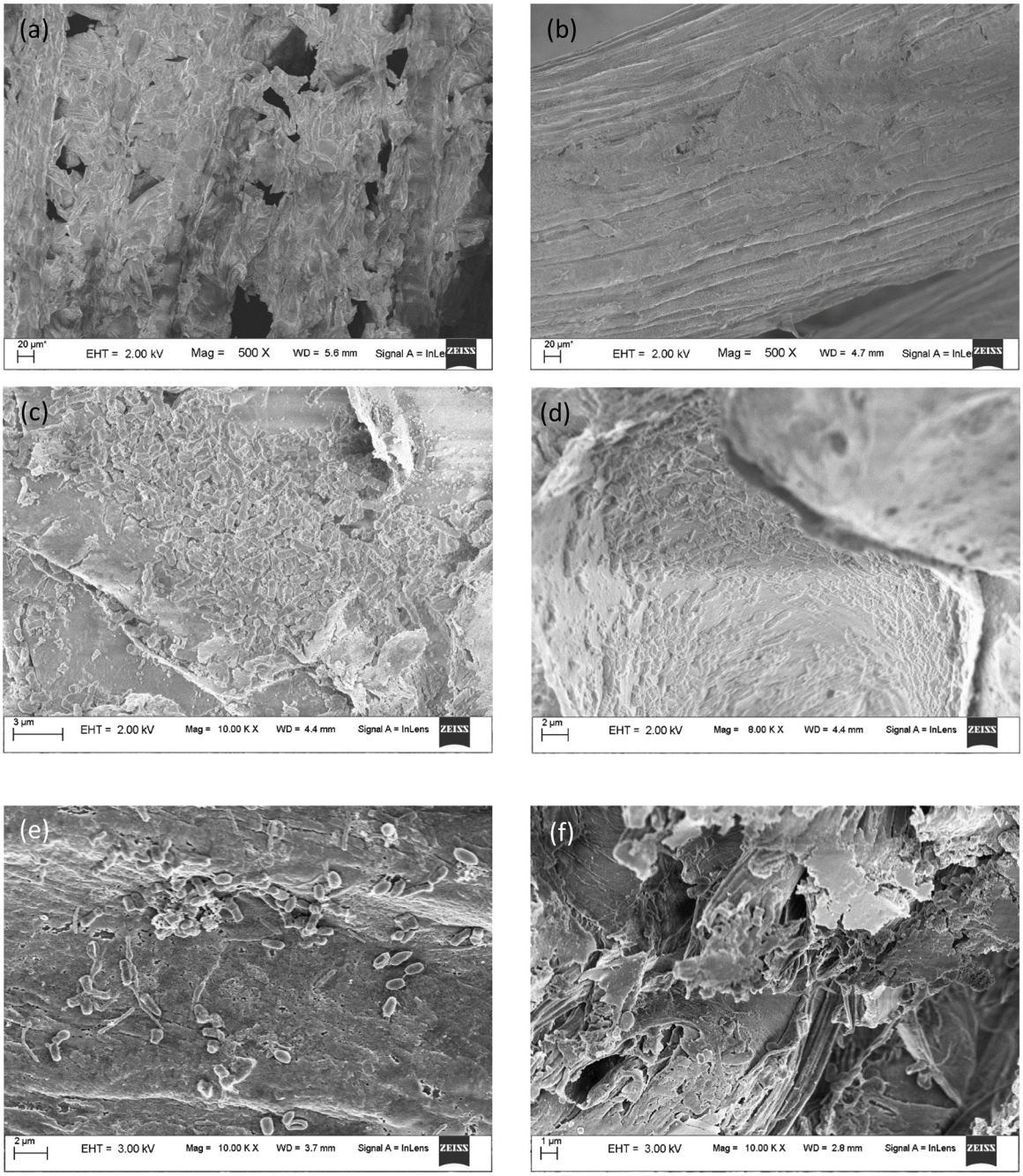

Fig. 1. (a) FESEM image of areca nut husk; (b) FESEM image of luffa sponge fiber; (c) & (d) Mixed bacterial culture immobilized on areca nut husks; (e) & (f) Mixed bacterial culture immobilized on luffa sponge fibers.

degradation rate was lower at a value of 0.23 h^{-1}. It may be noted that when compared to the suspension cell culture reported in our previous study [24], there is a marked improvement in phenol degradation. Phenol was degraded completely in pH values of 5.0, 6.0, 7.0 and 8.0. Besides, more than 99% of phenol was also degraded at pH 9 and pH 10 during the study duration. Whereas, in our previous study using free cells [24], at pH 5.5 and pH 8.5, a severe inhibition of growth was observed which indicated minimal or no degradation of phenol.

Similarly, Fig. 3a represents the phenol degradation curves at different pH values in luffa-sponge immobilization system. Here, it can be observed that 200 mg L^{-1} of phenol was completely degraded at pH 8.0 in 8 h. At pH 7.0, complete degradation was achieved in 10 h. Complete phenol degradation for pH 9.0 and pH 10.0 was achieved at 12 h and 14 h respectively. At pH 6.0, complete degradation of phenol took 20 h. However, at pH 5.0, phenol was not completely degraded in the duration of the study, and about 79% degradation could be achieved in 24 h. Fig. 3b represents the phenol degradation rates and corresponding degradation percentages for luffa immobilization system. The phenol degradation rate attained a maximum value of 0.21 h^{-1} at pH 8.0.

Hence, an initial pH of 8.0 was determined to be optimum for efficient phenol degradation in both the immobilization systems, since it was at this pH value that maximum phenol degradation rates were attained. A substantial improvement of the capability of immobilized bacterial cells in tolerating harsher pH conditions can be attributed to the fact that, immobilization on the lignocellulosic supports provided ample resistance and protection [13]. Moreover, immobilization makes larger number of bacterial cells available for utilization of substrate compared to the freely suspended counterparts which were not able to

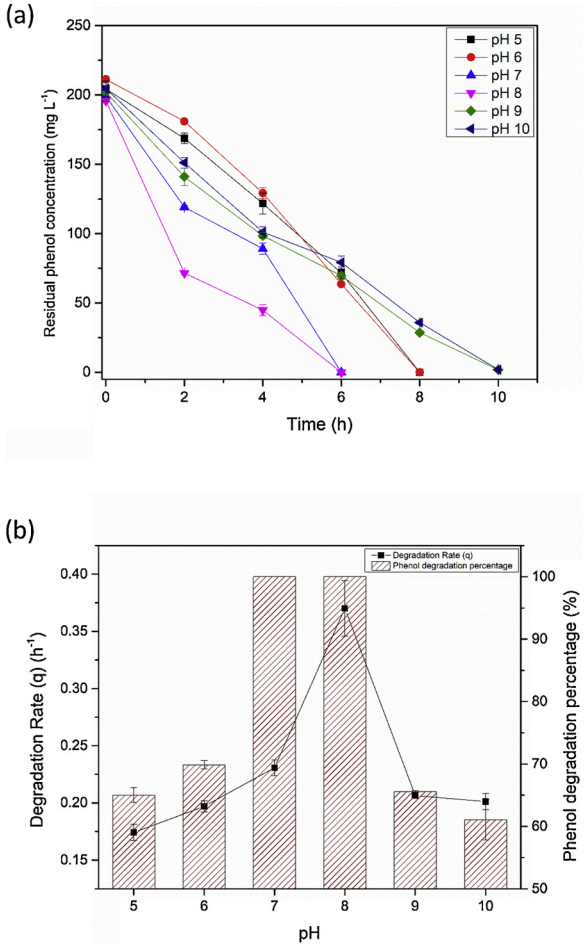

Fig. 2. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different pH values for areca nut husk system (Phenol = 200 mg L^{-1}; Temperature = 37 °C for an experimental duration of 6 h).

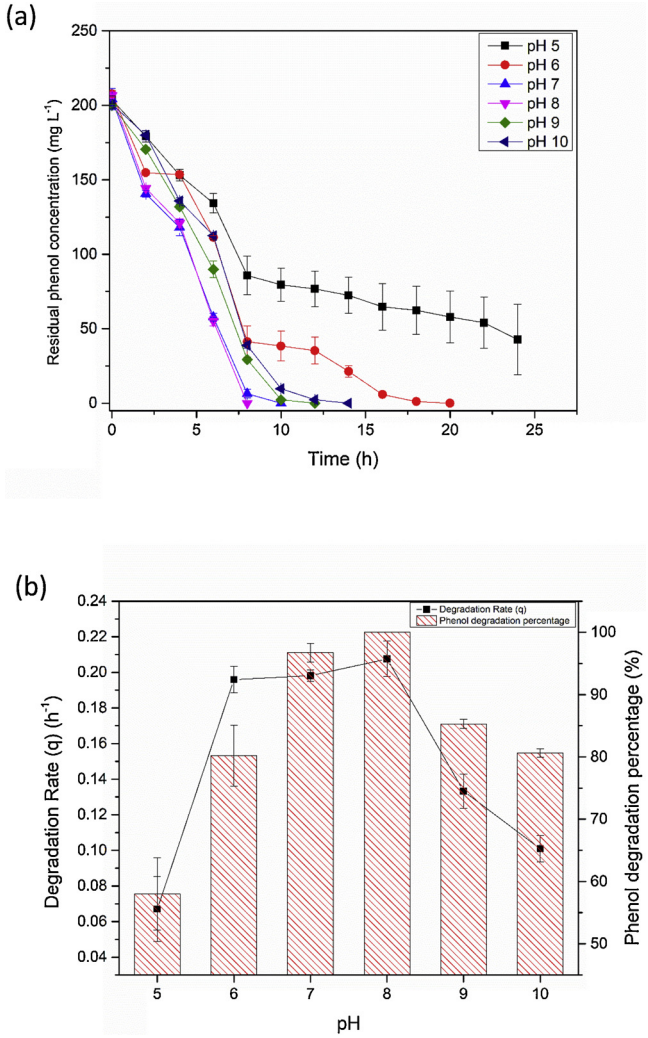

Fig. 3. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different pH values for luffa sponge system (Phenol = 200 mg L^{-1}; Temperature = 37 °C for an experimental duration of 8 h).

tolerate highly acidic or alkaline pH [24]. Areca nut husk system was able to tolerate and degrade phenol over a wider range of pH when compared to luffa sponge system. This could be attributed to the fact that the luffa sponges were less porous than areca nut husks and more bacterial cells were immobilized on the areca nut husks compared to luffa sponges. The surface morphology and porosity of the husks possibly provided superior protection to the bacterial cells compared to the luffa sponges, enabling the bacterial cells to tolerate harsher conditions.

3.3. Effect of incubation temperature on phenol bioremediation

Enzymes responsible for phenol degradation are vulnerable to temperature changes. Hence, an optimum temperature must be maintained for efficient utilization of a substrate. Suboptimal temperatures might lead to impaired functioning of enzymes, eventually slowing down organismal metabolism, leading to slower and inefficient consumption of phenol. Fig. 4a and Fig. 4b represents the degradation rates and degradation percentages of phenol at different temperatures of incubation for areca nut husk and luffa-sponge immobilization systems respectively. All experiments were performed at pH 8.0. Both cases, witnessed an improvement in degradation rate with rise in temperature. A peak degradation rate was achieved at 37 °C for both the immobilization systems. A maximum degradation rate of 0.37 h^{-1} was observed in case of areca nut husks, whereas, the maximum degradation rate was 0.21 h^{-1} in case of luffa sponges. Complete phenol degradation was achieved in 6 h and 8 h for areca nut husks and luffa sponge immobilization systems respectively. The degradation rate declined at 45 °C. In both cases, tolerance towards temperatures on either side of the optimum point was quite remarkable. For both immobilization systems, percentage degradation and degradation rate of phenol was more at 45 °C compared to 25 °C and 30 °C. Areca nut husk system could degrade 71.34% of phenol at 45 °C during the experimental duration (data not shown here). Whereas, luffa fiber system could achieve 65.18% phenol degradation at 45 °C (data not shown here). Phenol degradation rates at 30 °C for both immobilization systems were also acceptable. However, the degradation percentages of phenol at 25 °C was very low suggesting very slow substrate utilization rates. When compared to our previous study involving free cells [24], there was a remarkable improvement in phenol degradation at 45 °C. The mixed culture in free suspension exhibited a stunted growth featuring very low growth rates and almost no phenol degradation [24]. The above results verified that immobilization of microorganisms improved tolerance towards temperature changes.

3.4. Effect of initial substrate concentration on the degradation of phenol

Initial concentration of phenol plays a crucial role in the bioremediation process, as phenol is a very potent growth inhibitor. Experiments were carried out for both lignocellulosic biomass with different initial phenol concentrations (200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}) at an optimized pH and

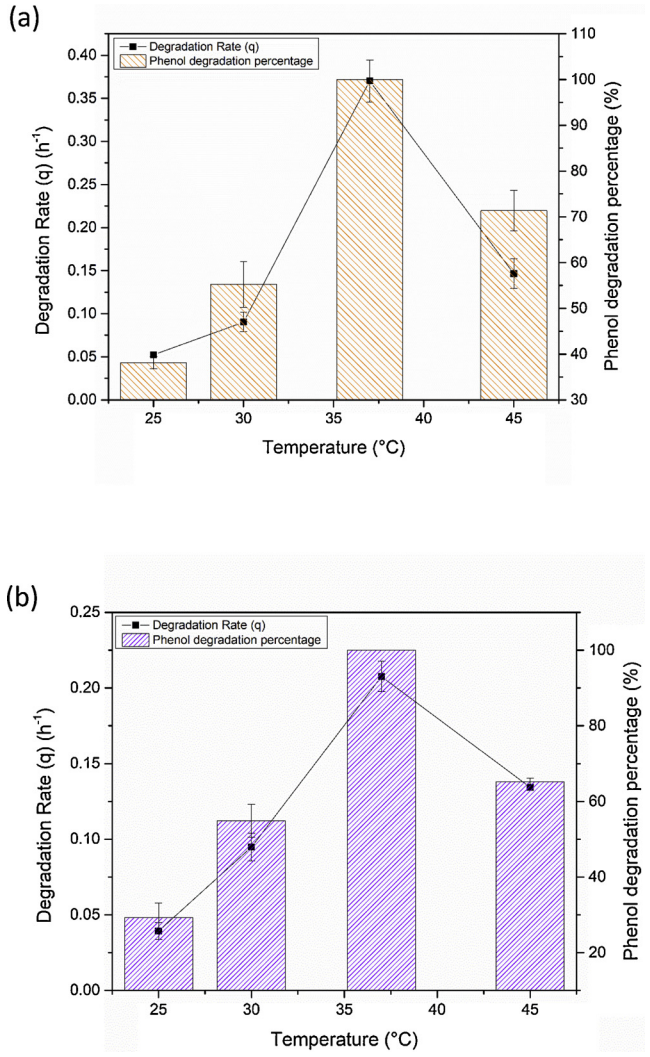

Fig. 4. (a) Phenol degradation rates and degradation percentages @ different temperatures for areca nut husk system (Phenol = 200 mg L^{-1}; pH = 8.0 for an experimental duration of 6 h); (b) for luffa sponge system (Phenol = 200 mg L^{-1}; pH = 8.0 for an experimental duration of 8 h).

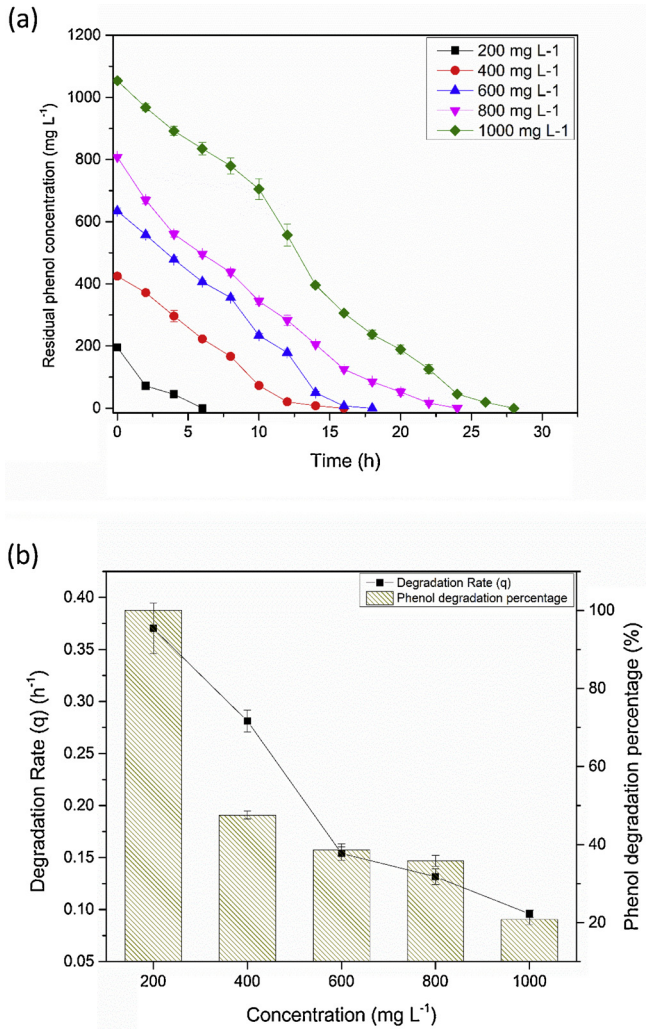

Fig. 5. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different initial phenol concentrations for areca nut husk system (pH = 8.0; Temperature = 37 °C for an experimental duration of 6 h).

temperature of 8.0 and 37 °C respectively.

Fig. 5a represents the phenol degradation profiles at various initial phenol concentrations for the areca nut husk system. Fig. 5b represents the phenol degradation rates and degradation percentages for the same. The maximum phenol degradation rate (0.37 h^{-1}) was observed at 200 mg L^{-1} phenol. The degradation rate declined with increase in phenol concentrations indicating the inhibitory effect of the substrate. The degradation rate at 1000 mg L^{-1} phenol was 0.096 h^{-1} and the areca nut husk immobilized microbes could degrade only about 21% of the initial substrate concentration. However, the experiment was continued to monitor the time taken for the depletion of phenol entirely in all the batches considered in the study. 1000 mg L^{-1} of phenol was completely depleted in 28 h.

Likewise, Fig. 6a depicts the phenol degradation profiles for the luffa sponge immobilization system at varying phenol concentrations. Fig. 6b represents the phenol degradation rates and degradation percentages for the luffa immobilized organisms. The maximum degradation rate was 0.21 h^{-1} and it was achieved by the organisms growing in 200 mg L^{-1} phenol. The trend of decline of the degradation rates were similar to the areca nut husk immobilization system indicating substrate inhibition. Organisms exhibited a minimum degradation rate of 0.089 h^{-1} at 1000 mg L^{-1} of phenol. However, 1000 mg L^{-1} phenol was degraded completely in 30 h by the microbes in the luffa sponge system.

The above results when compared to free cell culture from our earlier reported study revealed promising results. A free suspension of the mixed culture could degrade 1000 mg L^{-1} phenol in 96 h [24]. For both our immobilization systems, there is a remarkable improvement in phenol degradation times. The phenol degradation times reduced by more than three times in both cases. This enhancement of degradation can be attributed to the fact that immobilization shields the microorganisms from the toxic effects of phenol [6]. Moreover, reports also suggest that immobilization of bacterial cells enhance cellular activity by altering their physiological features in metabolism such as amplified enzyme induction, reduced specific cell growth and cell yield [3,6].

3.5. Modeling and kinetics of phenol degradation

Phenol degradation kinetics by the bacterial consortium immobilized on areca nut husks and luffa sponges was studied at different starting phenol concentrations (S_o) (200–1000 mg L^{-1}) of the growth medium. For each initial phenol concentration, experimental substrate degradation rates (q, h^{-1}) were calculated as per the description in Section 2.6. A maximum experimental degradation rate was achieved at a pollutant concentration of 200 mg L^{-1} for both areca nut husk and

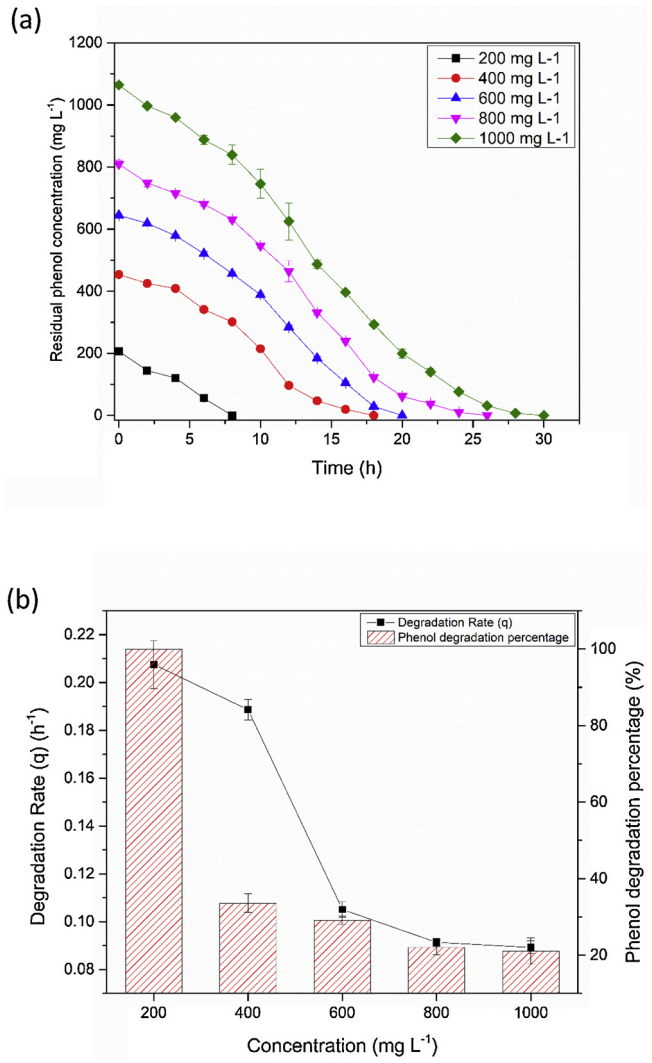

Fig. 6. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different initial phenol concentrations for luffa sponge system (pH = 8.0; Temperature = 37 °C for an experimental duration of 8 h).

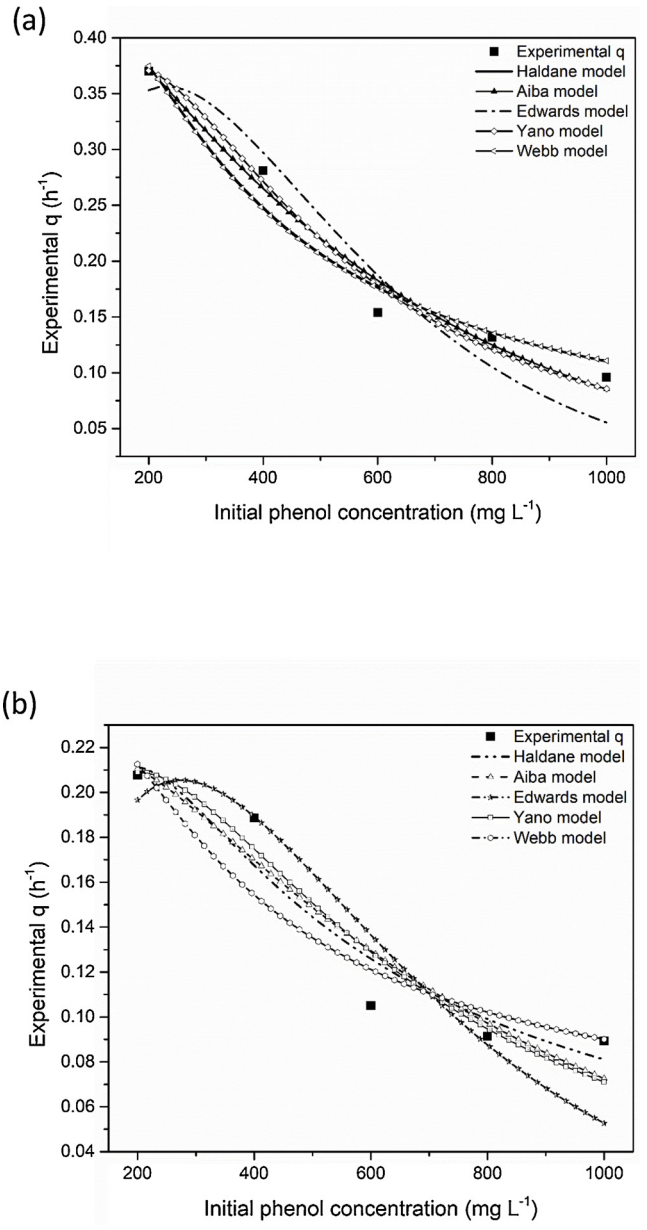

Fig. 7. Model prediction and fitting of experimental degradation rates at various initial phenol concentrations in: (a) areca nut husk system; (b) luffa sponge system (pH = 8.0; Temperature = 37 °C).

luffa sponge immobilization systems. The experimental degradation rates were comparable to the reported literatures which made use of synthetic matrices (Table 1). Thereafter, the degradation rates started declining, indicating substrate inhibition. The plot of experimental degradation rates versus their corresponding initial substrate concentrations was fitted to several available inhibition kinetic models via MATLAB R2017b. Fig. 7a and Fig. 7b depicts the fitting of experimental q with model simulated values of different inhibition models for areca nut husk and luffa sponge system respectively. Several bio-kinetic parameters were estimated by nonlinear regression analysis and portrayed in Table 2. The corresponding values of the coefficients of correlation (R^2) are also reported. The experimental degradation rates demonstrated by the mixed bacterial culture immobilized on the areca nut husks (0.37 h^{-1}) were found to be higher than the bacteria immobilized on luffa sponges (0.21 h^{-1}). Based on the value of correlation coefficient (R^2 = 0.9858) the best fit in case of areca nut husk system was given by Edward model with a predicted maximum degradation rate (q_{max}) of 0.0042 h^{-1}. The values of K_S and K_I were predicted to be 3.58 mg L^{-1} and 231 mg L^{-1} respectively. In case of luffa fibers, however, the best-fitted model was Aiba model with a R^2 value of 0.9903 and a q_{max} of 0.3413 h^{-1}. The values of K_S and K_I were calculated to be 39.5 mg L^{-1} and 663 mg L^{-1} respectively. The values of K_S points to the ability of the organisms to grow at a fairly higher concentration of phenol [13]. Apart from this, however, other models also fitted the experimental data fairly well in case of both the immobilization systems (owing to the value of R^2). Interestingly, Haldane model, which is the most widely used inhibition model, exhibited a very low value of K_I for both the immobilization systems (although R^2 is > 0.9). K_I value indicates inhibitory concentration of phenol, which is toxic to the organisms [13] and an inferior predicted K_I value (compared to K_S value) renders the Haldane model unsuitable.

3.6. Storage and reusability of the immobilized microorganisms

Stability of the immobilized cells during long-term storage is an indispensable aspect to be considered for the feasible application of the immobilized cell system [6]. The effect of storage of the immobilized microorganisms at 4 °C on degradation of phenol was studied for a period of 6 weeks. Both lignocellulosic biomass (with microorganisms immobilized) were stored for a period of 7, 14, 21, 28, 35 and 42 days

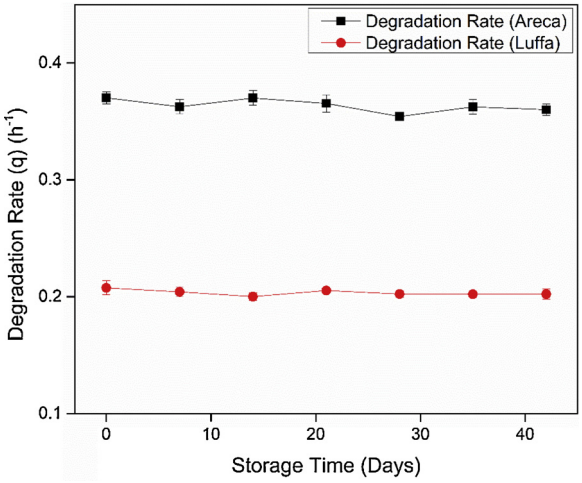

Fig. 8. Degradation rates of the immobilized bacterial culture when stored @ 4 °C for 0, 7, 14, 21, 28, 35 & 42 days (for both areca nut husk and luffa sponge systems).

Table 3
Compositional analysis of lignocellulosic biomass.

Biomass (without bacteria)	Hemicellulose content (wt%)	Cellulose content (wt%)	Lignin content (wt%)
Areca nut husk	26.8 ± 2.5	44.4 ± 1.0	14.0 ± 0.5
Luffa fibres	8.7 ± 1.2	65.1 ± 1.5	6.5 ± 1.6
Biomass (after 15 repeated batch experiments)			
Areca nut husk	24.5 ± 2.2	46.65 ± 1.3	16.2 ± 1.4
Luffa fibres	8.1 ± 1.5	63.05 ± 1.2	7.2 ± 0.7

at 4 °C. Fig. 8 portrays the variation of the phenol degradation rates of areca nut husk and luffa sponge systems at optimal degradation conditions for 200 mg L^{-1} phenol. It was noticed that there were minimal changes in degradation rates over the course of 6 weeks. The time taken for complete depletion of 200 mg L^{-1} of phenol was also noted for each batch. It was observed that, each batch of experiments took precisely 6 h and 8 h for complete degradation of 200 mg L^{-1} phenol (degradation data not shown here) for areca nut husk and luffa sponge supports respectively. These results demonstrated that the immobilized cells had a very strong storage stability and could successfully be reused for a long time. The obtained results were at par with earlier reported studies [6,7,13]. It is worth mentioning that the storage of immobilized cells is possible in sterilized glass containers or flasks at 4 °C without any special care and any significant deterioration of degradation efficiency.

Similar to storage, the stability of immobilized cells in long-term operation is also an important aspect, which must be considered for practical applicability of the immobilized cell system [3]. In order to investigate this, areca nut husk and luffa sponge supports containing the immobilized microbes were exposed to 15 consecutive batch degradation experiments. Each batch consisted of 200 mg L^{-1} of phenol and, 6 h and 8 h of incubation for areca nut husk and luffa sponge supports respectively. It was observed that there were negligible shifts in the degradation efficiencies in case of both the supports (data not shown here). Moreover, 200 mg L^{-1} of phenol was biodegraded completely in the duration of the experiment for each batch. The results are comparable to earlier reported literatures [6,13] and better than a few others [5,7]. Structural integrity in terms of lignocellulosic biomass composition was studied after the completion of reusability studies (results discussed in the next Section 3.7.).

3.7. Studies on compositional analysis of the lignocellulosic biomass

Compositional analysis studies were carried out to determine the degradation of structural components of the lignocellulosic biomass (if any) due to repeated batch degradation experiments. Table 3 summarizes the results of the compositional analysis studies. It can be clearly observed that there were no noticeable shifts in the structural composition of both the lignocellulosic biomass even after 15 consecutive batch biodegradation experiments. These results indicate the resilience of both the lignocellulosic biomass and their resistance towards degradation by the mixed culture that was immobilized on them. Hence, it can be concluded that both lignocellulosic biomasses can be used successfully as immobilization supports for the mixed bacterial culture to achieve effective and enhanced phenol degradation efficiencies.

4. Conclusions

A bacterial consortium was isolated from a petroleum refinery sludge in a previously reported study [24]. The consortium could degrade 1000 mg L^{-1} of phenol in 96 h. This study investigated the potential of two lignocellulosic biomass as possible candidates for immobilization of the mixed bacterial culture for enhanced phenol remediation. We have used dry areca nut husks and mature luffa sponges as immobilization supports. The mixed bacterial culture was immobilized on the supports via natural adsorption. When compared to the freely suspended degradation system (reported in our earlier study) [24], the immobilized system could degrade phenol a lot faster under optimized pH and temperature conditions. The optimum pH and temperature for efficient phenol degradation were determined to be 8.0 and 37 °C respectively. The enhanced tolerance of the immobilized systems towards adverse conditions have also been demonstrated in this study. Degradation kinetic studies were carried out using different substrate inhibition models and bio-kinetic parameters were estimated via non-linear regression analysis in MATLAB. The best-fitted model for areca nut husk and luffa sponge system was Edward model and Aiba model respectively. Interestingly, the immobilized microorganisms could be stored at 4 °C for up to 6 weeks without any noticeable loss in degradation efficiency. The immobilized microorganisms could also be used for successfully conducting up to 15 consecutive batch biodegradation experiments. Compositional analysis studies of both the lignocellulosic biomass before immobilization and after 15 successive batch experiments revealed that the structural components of the biomass were not degraded, which establishes the fact that these can be successfully used for long-term operation of reactors. These results establish the fact that areca nut husks and luffa sponge fibers have the potential to be efficiently used as inexpensive immobilization matrices for the bioremediation of environmental pollutants. Further studies are being carried out to check the efficiency of these matrices in lab-scale packed bed reactors and will be reported in future communications.

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References

[1] B. Pradhan, S. Murugavelh, K. Mohanty, Phenol biodegradation by indigenous mixed microbial consortium: growth kinetics and inhibition, Environ. Eng. Sci. 29 (2011) 86–92, https://doi.org/10.1089/ees.2011.0024.
[2] H. Kumar, K. Mohanty, Kinetic modelling of phenol biodegradation by mixed microbial culture in static batch mode, Asian J. Water, Environ. Pollut. 9 (2012) 19–24.

[3] O. Ali, A. Namane, A. Hellal, Use and recycling of Ca-alginate biocatalyst for removal of phenol from wastewater, J. Ind. Eng. Chem. 19 (2013) 1384–1390, https://doi.org/10.1016/j.jiec.2012.12.045.
[4] S. Al-Zuhair, M. El-Naas, Immobilization of Pseudomonas putida in PVA gel particles for the biodegradation of phenol at high concentrations, Biochem. Eng. J. 56 (2011) 46–50, https://doi.org/10.1016/j.bej.2011.05.005.
[5] B. Basak, B. Bhunia, A. Dey, Studies on the potential use of sugarcane bagasse as carrier matrix for immobilization of candida tropicalis PHB5 for phenol biodegradation, Int. Biodeterior. Biodegrad. 93 (2014) 107–117, https://doi.org/10.1016/j.ibiod.2014.05.012.
[6] Y. Wang, Y. Tian, B. Han, H. Zhao, J. Bi, B. Cai, Biodegradation of phenol by free and immobilised Acinetobacter sp, J. Environ. Sci. 19 (2007) 222–225.
[7] A. Banerjee, A.K. Ghoshal, Phenol degradation performance by isolated Bacillus cereus immobilized in alginate, Int. Biodeterior. Biodegrad. 65 (2011) 1052–1060, https://doi.org/10.1016/j.ibiod.2011.04.011.
[8] A. Banerjee, A.K. Ghoshal, Biodegradation of phenol by calcium-alginate immobilized @@ Bacillus cereus in a packed bed reactor and determination of the mass transfer correlation, J. Environ. Chem. Eng. 4 (2016) 1523–1529, https://doi.org/10.1016/j.jece.2016.02.012.
[9] M. Mollaei, S. Abdollahpour, S. Atashgahi, H. Abbasi, F. Masoomi, I. Rad, A.S. Lotfi, H.S. Zahiri, H. Vali, K.A. Noghabi, Enhanced phenol degradation by Pseudomonas sp. SA01: Gaining insight into the novel single and hybrid immobilizations, J. Hazard. Mater. 175 (2010) 284–292, https://doi.org/10.1016/j.jhazmat.2009.10.002.
[10] Y.M. Chen, T.F. Lin, C. Huang, J.C. Lin, F.M. Hsieh, Degradation of phenol and TCE using suspended and chitosan-bead immobilized Pseudomonas putida, J. Hazard. Mater. 148 (2007) 660–670, https://doi.org/10.1016/j.jhazmat.2007.03.030.
[11] C.H. Chen, L.M. Whang, C.L. Pan, C.L. Yang, P.W.G. Liu, Immobilization of diesel-degrading consortia for bioremediation of diesel-contaminated groundwater and seawater, Int. Biodeterior. Biodegrad. 124 (2017) 62–72, https://doi.org/10.1016/j.ibiod.2017.07.001.
[12] H. Keweloh, H.J. Heipieper, H.J. Rehm, Protection of bacteria against toxicity of phenol by immobilization in calcium alginate, Appl. Microbiol. Biotechnol. 31 (1989) 383–389, https://doi.org/10.1007/BF00257609.
[13] Y.J. Liu, A.N. Zhang, X.C. Wang, Biodegradation of phenol by using free and immobilized cells of Acinetobacter sp. XA05 and Sphingomonas sp. FG03, Biochem. Eng. J. 44 (2009) 187–192, https://doi.org/10.1016/j.bej.2008.12.001.
[14] M.K. Kureel, S.R. Geed, B.S. Giri, B.N. Rai, R.S. Singh, Biodegradation and kinetic study of benzene in bioreactor packed with PUF and alginate beads and immobilized with Bacillus sp, M3, Bioresour. Technol. 242 (2017) 92–100, https://doi.org/10.1016/j.biortech.2017.03.167.
[15] J.H. Kim, K.K. Oh, S.T. Lee, S.W. Kim, S.I. Hong, Biodegradation of phenol and chlorophenols with defined mixed culture in shake-flasks and a packed bed reactor, Process Biochem. 37 (2002) 1367–1373, https://doi.org/10.1016/S0032-9592(02)00007-9.
[16] M. Bajaj, C. Gallert, J. Winter, Biodegradation of high phenol containing synthetic wastewater by an aerobic fixed bed reactor, Bioresour. Technol. 99 (2008) 8376–8381, https://doi.org/10.1016/j.biortech.2008.02.057.
[17] J. Yu, X. Zhang, T. Tan, An novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production, J. Biotechnol. 129 (2007) 415–420, https://doi.org/10.1016/j.jbiotec.2007.01.039.
[18] S. Wang, X. Li, W. Liu, P. Li, L. Kong, W. Ren, H. Wu, Y. Tu, Degradation of pyrene by immobilized microorganisms in saline-alkaline soil, J. Environ. Sci. (China) 24 (2012) 1662–1669, https://doi.org/10.1016/S1001-0742(11)60963-7.
[19] S. Sasmal, V.V. Goud, K. Mohanty, Optimisation of the acid catalysed pretreatment of areca nut husk fibre using the Taguchi design method, Biosyst. Eng. 110 (2011) 465–472, https://doi.org/10.1016/j.biosystemseng.2011.09.013.
[20] G. Narayanamurthy, Y.L. Ramachandra, S. Padmalatha Rai, Y.N. Manohara, B.T. Kavitha, Areca husk: an inexpensive substrate for citric acid production by Aspergillus niger under solid state fermentation, Indian J. Biotechnol. 7 (2008) 99–102.
[21] S. Behera, R.C. Mohanty, R.C. Ray, Ethanol production from mahula (Madhuca latifolia L.) flowers with immobilized cells of Saccharomyces cerevisiae in Luffa cylindrica L. sponge discs, Appl. Energy 88 (2011) 212–215, https://doi.org/10.1016/j.apenergy.2010.07.035.
[22] J.C. Ogbonna, S. Tomiyama, Y.C. Liu, H. Tanaka, Efficient production of ethanol by cells immobilized in loofa (Luffa cylindrica) sponge, J. Ferment. Bioeng. 84 (1997) 271–274, https://doi.org/10.1016/S0922-338X(97)82069-7.
[23] D.V. Sriharsha, L. Kumar R, J. Savitha, Immobilized fungi on Luffa cylindrica: an effective biosorbent for the removal of lead, J. Taiwan Inst. Chem. Eng. 80 (2017) 589–595, https://doi.org/10.1016/j.jtice.2017.08.032.
[24] S. Bera, A.S. Roy, K. Mohanty, Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site, Int. Biodeterior. Biodegrad. 121 (2017) 107–113, https://doi.org/10.1016/j.ibiod.2017.04.002.
[25] J.B.S. Haldane, Enzymes, MIT Press, Cambridge, MA, 1965.
[26] T. Yano, T. Nakahara, S. Kamiyama, K. Yamada, Kinetic studies on microbial activities in concentrated solutions, Agric. Biol. Chem. 30 (1966) 42–48, https://doi.org/10.1080/00021369.1966.10858549.
[27] S. Aiba, M. Shoda, M. Nagatani, Kinetics of product inhibition in alcohol fermentation, Biotechnol. Bioeng. 10 (1968) 845–864, https://doi.org/10.1002/bit.260100610.
[28] V.H. Edwards, The influence of high substrate concentrations on microbial kinetics, Biotechnol. Bioeng. 12 (1970) 679–712, https://doi.org/10.1002/bit.260120504.
[29] J.L. Webb, Enzyme and Metabolic Inhibitors, Academic Press, Boston, USA, 1963.
[30] H.K. Goering, P.J.V. Soest, Forage Fiber Analysis (USDA-ARS Agriculture Handbook 379), Gov. Print. Off., Washington, DC, 1970.
[31] L.E. Wise, Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses, Pap. Trade. 122 (1946) 35–43.
[32] E. Kurzbaum, Y. Raizner, O. Cohen, R.Y. Suckeveriene, A. Kulikov, B. Hakimi, L. Iasur Kruh, R. Armon, Y. Farber, O. Menashe, Encapsulated Pseudomonas putida for phenol biodegradation: use of a structural membrane for construction of a well-organized confined particle, Water Res. 121 (2017) 37–45, https://doi.org/10.1016/j.watres.2017.04.079.