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Application of the Principal Component Analysis (PCA) to aerobic biodegradation process

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Abstract

The Principal Component Analysis (PCA) is a method widely used to process experimental data. The PCA aims to reduce the dimensionality of a data set composed of many interrelated variables, preserving the variation as much as possible. This can be achieved by transforming the data set to a new one, containing the principal components (PCs), which are uncorrelated, and which maximize the variance of the original data. In this study, the PCA was applied to experimental tests on aerobic bioremediation of soil polluted with diesel oil. Twelve microcosms (200 g of soil) were prepared, polluted with commercial diesel oil (70 g/kg of dry soil), and hydrated with a mineral salt solution suitable to stimulate the indigenous bacteria. The aerobic process for diesel oil degradation was studied changing two operative parameters, namely, water content (u%) and carbon to nitrogen ratio (C/N). Three values of water content (u% = 8%, 12% and 15% by weight) and four of carbon to nitrogen ratio (C/N = 60, 120, 180 and 300) were tested. The microcosms were monitored for 30 days by the measurements of these parameters: 1) the CO_2 production, to evaluate the microbial respiration, 2) the fluorescein production, to check the microbial activity, and 3) the diesel oil concentration, to assess the pollutant degradation and calculate the removal efficiency. The Principal Component Analysis was done considering the water content values as variables and the carbon to nitrogen ones as systems to be analyzed. By a linear transformation, two principal components were achieved, namely PC1 and PC2. There sults for the CO_2 production, fluorescein production, and diesel oil removal efficiency were used, and overall variances always over 94% were obtained. The results showed that two microcosms stood out from the others, namely:-the microcosm with u% = 12% b.w. and C/N = 180,sinceit gave the highest amount of CO_2 and fluorescein;- the microcosm with u% = 8% b.w. and C/N = 120,due to the highest diesel oil removal efficiency.

Keywords: Principal component analysis (PCA), aerobic bioremediation, diesel oil-contaminated soil, pollution removal efficiency.

Introduction

Very often, in experimental studies, the amount of data is very large, and their analysis and use become difficult. It is important to understand which are the measured quantities that characterize the studied phenomenon or which combination of the measured quantities describes it simply and completely. At the same time, one or more parameters can be linearly dependent on the others, and this means that they do not give additional information to the study. Thus, many times, it is compulsory to reduce the data dimensionality as much as possible, without disregarding the bulk of the information contained in the data (Jolliffe, 2002).

For this purpose, many statistical techniques have been developed, mainly as multivariate analysis. This analysis allows examining simultaneously all the characteristic variables of a set of data (Afifi et al., 2012). Among these techniques, the Principal Component Analysis (PCA) is one of the oldest and most widely used (Jolliffe and Cadima, 2015). The PCA allows for to reduction of the dimensionality of a data set with a linear

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transformation. The new variables, called Principal Component (PCs), are uncorrelated and constitutes a linear combination of the original variables (Jolliffe, 2002). The principal components are arranged by decreasing variance since the variance represents the information of the old variables. The most informative principal component is the first, and the least informative is the last (Afifi et al., 2012).

The field of application of PCA analysis is very broad, for example: in physics, to analyze trajectories generated by molecular dynamics (Shenai et al., 2012); in agronomy, to assess some chemical, agronomic and morphological traits of different vegetable varieties (Renna et al., 2019);in environmental engineering, to examine the analytical results of bioremediation process (Ceccanti et al., 2006), orto evaluate the most efficient bioindicator for pollution monitoring (Onwosi et al., 2019); in computer technology.

In the current study, the PCA was applied to bioremediation. In this process, the metabolic activity of the indigenous bacterial consortia was exploited to removes oil pollution. The process efficiency depends on several factors, extensively studied over the years, as water content, carbon to nitrogen ratio, temperature, type of soil, and type of pollutant(s).

The PCA was applied to the study as the change of the water content (u%) influences the biodegradation of diesel oil in contaminated soils. Four types of microcosms were considered, with different carbon to nitrogen ratios (C/N). The analysis was carried out considering the data of carbon dioxide production, fluorescein production, and diesel oil removal efficiency.

Materials and Methods

Principal component analysis (PCA)

The principal component analysis is based on matrix calculation. The aim is to reduce the dimensionality of set data through a linear transformation.

The experimental data are represented by a matrix X:

$$X = [N \times K]$$

whereN is the number of available samples and K is the number of variables used in the experimental study.

This multivariable statistical method can transform the data of X in a new vector space, whose directions are said principal components (PCs). When two or more original variables of matrix X are correlated, it is possible to identify a common direction of variability which can be defined by a single PC. The matrix X can be represented by a different number of principal components, lower than the number of studied variables. The PCs are determined in such a way as to maximize the variability of the original data.

For this reason, the PCA method aims to perform optimization byan analytical solution based on the calculation of the eigenvectors and eigen values of the covariance matrix of X.

For the analysis, only the eigenvectors of the covariance matrix with an eigen value higher than 1 are considered. Then, they are sorted in descending order, and the first eigenvector is the first principal component PC1, which constitutes the highest variance of the original data. The coefficients of the vector PC1 are called loadings, and they are the cosine directors of the first principal component. The second eigenvector is the

second principal component PC2, and their coefficients are the PC2 loadings. The projection of the original data along the PC direction is the vector of the scores.

To this point, the original matrix can be rewritten as:

$$X = \sum_{s=1}^{Z} t_s p_s^T + \sum_{s=Z+1}^{S} t_s p_s^T = TP^T + E$$

Where T = [t_1, t_2,…,t_Z] is the scores matrix, P = [p_1, p_2,…,p_Z] is the loadings matrix, E is the residues matrix. The residues matrix reflects the variability of the data that is not represented by the model (Valle et al., 1999). It often happens that the original data are characterized by different units of measurement and they are not comparable. For this reason, the original data are standardized by subtracting from each element the mean of the respective column, and the result is divided by the standard deviation of the same column.

The score and loading values are shown in the score and loadings plots. To clarify, the score plot reflects the differences between the experimental tests, while the loadings plot shows how each variable influences a principal component. The score and loadings are shown in a single plot, called biplot, where:

- The abscissa axis reports the first principal component, PC1,and the ordinate axis reports the second principal component, PC2.
- The cosine of the angle between any vector representing a variable and the axis representing a given PC is the correlation coefficient between those two variables, called the loading.
- The points of the plot are the scores, to say the coordinates of the original data in the new reference system.

On the score plot, the distance among the points gives information on the similarity of the operative parameters: close points mean similar operative parameters, whereas the data are far if they were achieved with different operative conditions. The position of a variable on the score plot reflect show much this variable is correlated with the components, and the distance from the origin indicates the explained variance of this correlation. The confidence levels of explained variance can be drawn through ellipses; usually, the 95%confidence level is given, even if this level is not a rule (Jolliffe, 2002).

On the loading plot, the variables are shown as vectors (Jolliffe, 2002):

- The high correlation between two variables leads to two vectors that are very close (their angle is lower than 90°).
- The non-correlation leads to two vectors with an angle greater than 90°).
- The anti-correlation leads to two vectors with an angle close to 180°.

The biplot joins information of both plots. On it, the range of axes is -1/1, and the points of the experimental tests reproduce the position on the score plot, but on a different scale.

Soil microcosms and analytical measurements

The bioremediation process was studied deeply, and previous papers reported the findings (Vergnano et al., 2019; Raffa et al., 2020). In these publications, the microcosms preparation and the analytical measurements were described in detail. A general description of the studied bioremediation process is given below.

The aerobic biodegradation process was studied in 12microcosms, constituted with 200 g of soil, and polluted with diesel oil (70 g/kg of dry soil). The microbial growth was stimulated with a mineral salt solution suitable for bacteria (MSMB), dosed to get the appropriate C/N ratio and water content (Raffa et al., 2020).

Four values of carbon to nitrogen ratio were tested: C/N = 60, 120, 180, and 300, to assess the biodegradation process when the nutrient supply was changed. Three values of water content were adopted: u% = 8%, 12% and 15% by weight.

The aerobic process was monitored for 30 days, by:

- the CO_2 production, to assess the microbial respiration;
- the fluorescein production, to check the microbial activity: the fluorescein diacetate (FDA) is hydrolyzed by enzymes active in the biodegradation process, and fluorescein is the final product;
- the diesel oil concentration, to check the diesel oil degradation and evaluate the diesel oil removal efficiency as:

$$\eta = \frac{c_{IN} - c_{FIN}}{c_{IN}}$$

With c_{IN} and c_{FIN} the diesel oil concentration at t = 0 and t = 30 days, respectively.

All the analytical methods can be found in Raffa et al. (2020).

Results

The values used for the PCA were measured on the 30[th]day. For each variable, 12data were collected, shown in Table 1 (CO_2 production), Table 2 (fluorescein production), and Table 3 (diesel oil removal efficiency), respectively. For each data set, the PCA was done on standardized variables.

According to the PCA, the linear transformation was done by calculating the covariance matrix for each data set, and then their eigenvectors and eigen values.

The principal components derive from a linear transformation of the original data; thus, looking at the coefficients of vectors (the loadings), it is possible to evaluate which are the original variables that weigh more in the new ones. The score values give the correlation of tested systems with the principal components: high positive values indicate a positive correlation, high negative values indicate a negative correlation, values close to zero indicate a low correlation. In this way, the dimensionality of the original data can be reduced.

Table 1:Carbon dioxide production

CO_2 production (g/kg of dry soil)	Original variables			Standardized variables		
	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.
C/N = 60	3.6	2.9	1.9	0.6	-0.1	-1.3
C/N = 120	3.7	3.5	3.4	0.7	0.4	1.2
C/N = 180	3.3	4.3	2.8	0.2	1.0	0.2
C/N = 300	1.6	1.5	2.6	-1.5	-1.3	-0.1

Table 2: Fluorescein production

Fluorescein production (mg/kg of dry soil)	Original variables			Standardized variables		
	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.
C/N = 60	2.7	1.4	0.9	-0.8	-0.9	-1.0
C/N = 120	2.6	7.1	11.3	-0.8	0.2	1.4
C/N = 180	9.8	12.3	4.9	1.3	1.3	-0.1
C/N = 300	6.4	3.2	4.7	0.3	-0.6	-0.2

Table 3: Diesel oil removal efficiency

Diesel oil removal efficiency (%)	Original variables			Standardized variables		
	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.	u% = 8% b.w.	u% = 12% b.w.	u% = 15% b.w.
C/N = 60	5	3	6	-0.5	-0.9	0.0
C/N = 120	33	6	16	1.4	-0.6	1.4
C/N = 180	10	22	1	-0.2	1.3	-0.7
C/N = 300	1	13	2	-0.8	0.2	-0.7

In this study, the original variables were three, then reduced to two. The PC1 and PC2 allowed to reach a maximum variance of over 94.0%.

CO₂production

For the CO_2production, the vector coefficients of the principal components are shown in Table 4. The PC1 represents the 63.3% of the variance and is better represented by u% = 8% b.w and u% = 12% b.w, since the loadings (p_1) are 0.64 and 0.69, respectively. The PC2 represents the 31.0% of the variance and it is constituted by u% = 15% b.w with a loading (p_2) equal to 0.91.

Table 4: Loadings of CO_2production

Loadings	p₁	p₂
u% = 8% b.w.	0.64	-0.40
u% = 12% b.w.	0.69	-0.08
u% = 15% b.w.	0.34	0.91

The similar behavior of different microcosms can be seen comparing the scores (Table 5), and their representation in the new reference frame.

Table 5: Scores of CO_2production

Scores	t₁	t₂
C/N = 60	-0.13	-1.36
C/N = 120	1.07	0.81
C/N = 180	0.96	0.00
C/N = 300	-1.90	0.55

Table 5 shows that the microcosms with C/N = 120, 180, and 300 are better represented by the first principal component (t_1: 1.07, 0.96,-1.90), while the microcosms with C/N = 60 are characterized by the second principal component (t_2: -1.36).

The score values can be shown in the score plot (Figure 1): the points of microcosms with C/N = 120 and C/N = 180 are close, while the points of C/N = 60 and 300 are rather far. One reason can be the greater CO_2 quantities produced by the microcosms with C/N = 120 and C/N = 180 than by the microcosms with C/N = 60 and C/N = 300, especially with water contents equal to 8% and 12% by weight, respectively. Moreover, the microcosms with C/N = 120 have similar CO_2productionat different water contents, and the PC coordinates have similar values (t_1: 1.07,t_2: 0.81).

The maximum variance is equal to 94.3% and all the points fall within the confidence level.

Figure 2 shows the biplot for the CO_2 production data.The positive correlation between u% = 8% b.w. and u% = 12% b.w., and u% = 12% b.w. and u% = 15% b.w. is visible, due to α < 90°. The microcosms with C/N = 120 and 180 are influenced by the variable u% = 12% b.w..
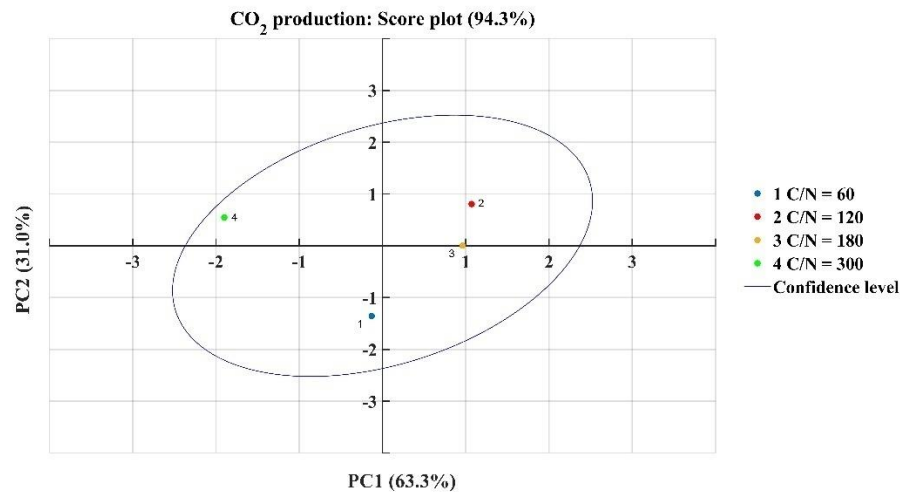
Figure 1.Score plot: CO_2 production with a 95% confidence level
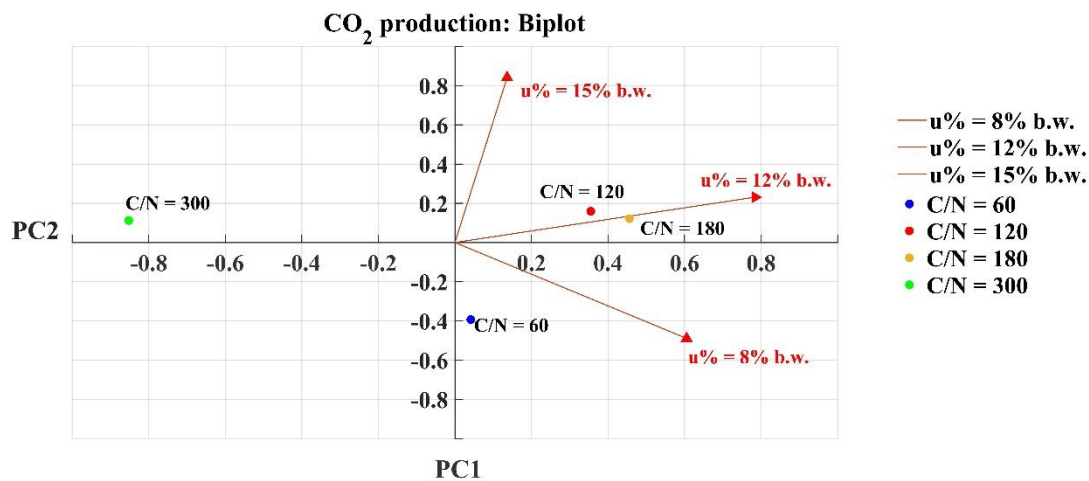
Figure 2. Biplot: CO_2 production

Fluorescein production

For the fluorescein production data set, the first principal component mainly depends on the variables u% = 8% b.w. and u% = 12% b.w. (loadings p_1 are equal to 0.62 and 0.73, respectively), and much less on the u% = 15% b.w. (loading p_2 equal to 0.27). The original data are represented by the first principal component with a variance of 58.1%.Then, the second principal component is represented by the variable u% = 15% b.w., and its variance is equal to 38.0%.

Table 6: Loadings of fluorescein production

Loadings	p_1	p_2
u% = 8% b.w.	0.62	-0.49
u% = 12% b.w.	0.73	0.10
u% = 15% b.w.	0.27	0.86

Looking at the scores of fluorescein production (Table 7), the microcosms with C/N = 60 and 180 are better described by the first principal component (t_1: -1.47, 1.73), the microcosms with C/N = 120 by the second principal component (t_2: 1.59), while the microcosms with C/N = 300 have not a prevalence of one component over the other (t_1: -0.29; t_2: -0.36).

Table 7: Scores of fluorescein production

Scores	t_1	t_2
C/N = 60	-1.47	-0.61
C/N = 120	0.04	1.59
C/N = 180	1.73	-0.62
C/N = 300	-0.29	-0.36

The points on the score plot (Figure 3) show as the microcosms with C/N = 180 are rather far from the other systems, and this could be explained by the high production of fluorescein. The prevalence of the second principal component in the microcosms with C/N = 120 could be due to the greater production of fluorescein with a water content equal to 15% b.w. than in other runs.
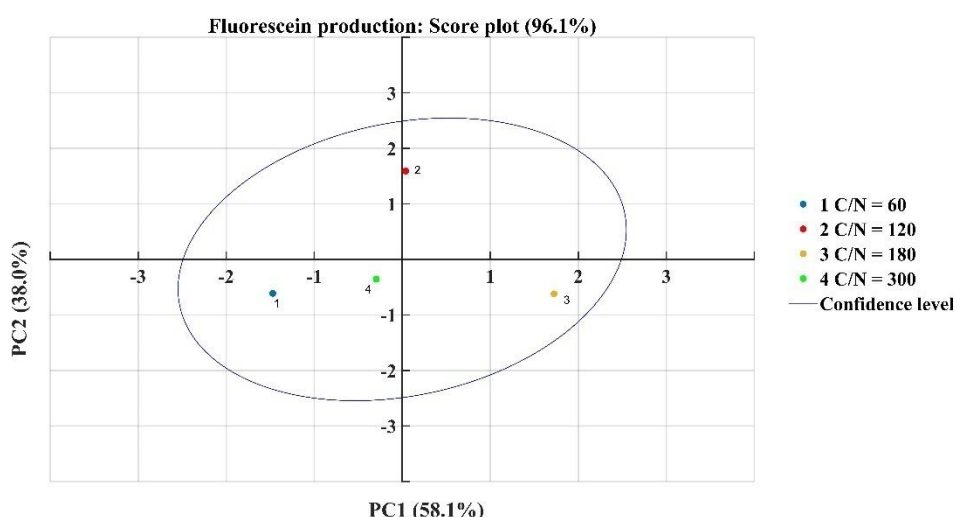
Figure 3.Score plot: Fluorescein productionwitha 95% confidence level

The PCA analysis is suitable for the tested data, the maximum variance is 96.1% and no points are out of the confidence level.

The biplot (Figure 4) encloses the information on scores and loadings. There isa positive correlation between the u% = 8% b.w. and u% = 12% b.w.. The microcosms with C/N = 120 are close to the vector of variable u% = 15% b.w, while the C/N = 180 is between the vectors of u% = 8% b.w. and 12% b.w..

Figure 4: Biplot: Fluorescein production

Diesel oil removal efficiency

The loadings of the principal components for the diesel oil removal efficiency(Table 8)show that the PC1 is represented by the variables with u% = 8% b.w. and u% = 15% b.w. (p_1: 0.58 and 0.67) and the variance of the original data is equal to 74.5%, while the PC2 is composed mainly by the variable with u% = 12% b.w. (p_2: 0.82). The PCs have a different meaning compared to the ones of CO_2 and fluorescein production, where the water contents equal to 8% b.w. and 12% b.w. were the variables better represented by the first principal component.
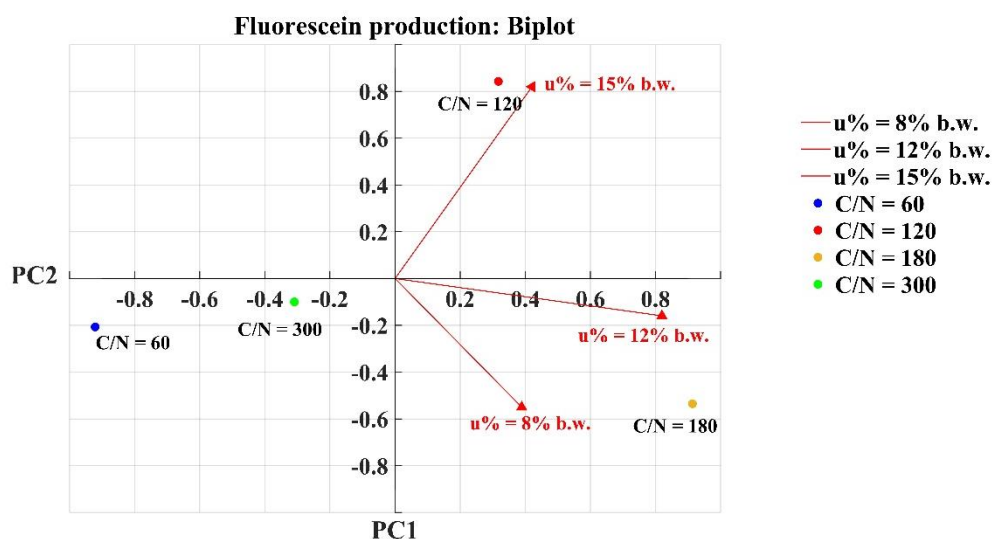
Table 8: Loadings of diesel oil removal efficiency

Loadings	p_1	p_2
u% = 8% b.w.	0.58	0.57
u% = 12% b.w.	-0.47	0.82
u% = 15% b.w.	0.67	0.08

In this case, the scores of the microcosms (Table 9) evidence the difference between the two principal components. One component always prevails over the other: for the microcosms with C/N = 120, 180, and 300 the PC1 prevails, while for the microcosms with C/N = 60, the PC2 does.

Table 9: Scores of diesel oil removal efficiency

Scores	t_1	t_2
C/N = 60	0.13	-1.07
C/N = 120	2.07	0.45
C/N = 180	-1.20	0.92
C/N = 300	-1.00	-0.30

The score plot (Figure 5) puts in evidence that the microcosms with C/N = 120 are different compared to the others: they have the highest diesel removal efficiency.
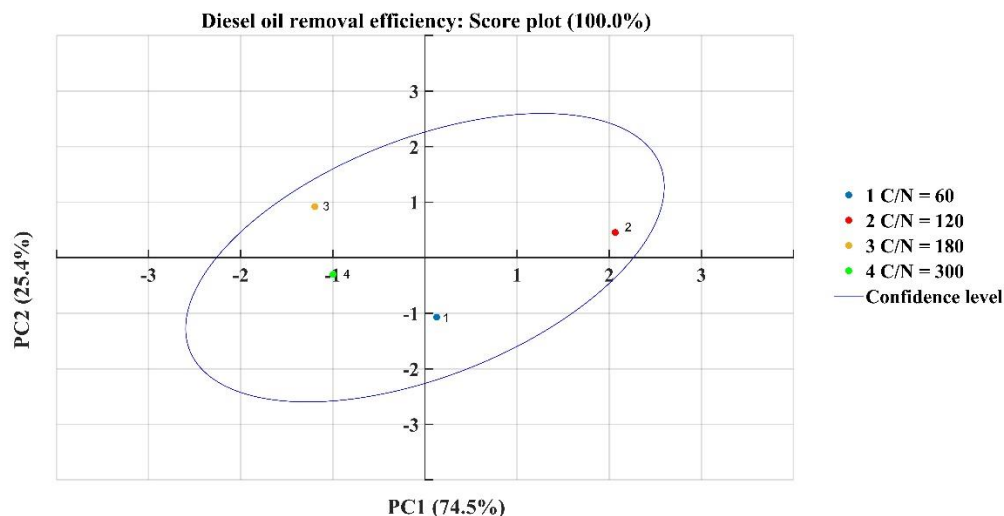
Figure 5.Score plot: Diesel oil removal efficiency with a 95% confidence level

For the diesel oil removal efficiency data, the reached maximum variance is equal to 100.0%, with the first principal component representing74.5% of the original data. There are no points outside the confidence level. Figure 6 reports the biplot for these results. The variables u% = 8% b.w. and 15% b.w. are non-correlate ($\alpha <$ 90°), while the correlation between u% = 8% b.w. and 15% b.w. is positive.
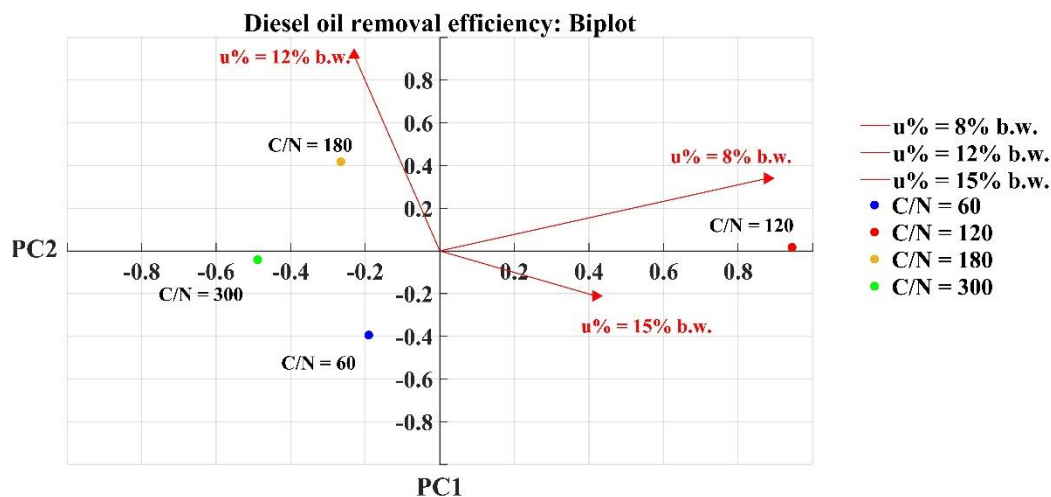
Figure 6. Biplot: Diesel oil removal efficiency

Conclusions

The principal component analysis applied to a bioremediation study was demonstrated to be suitable for fitting the experimental data, especially for the diesel oil removal efficiency. The analysis showed that the studied process can be evaluated considering only two variables.

The main results of PCA showed that:

1. the principal components gave the maximum variance always over 94%;

2. for the CO_2 and fluorescein production, the PC1 was influenced by water contents equal to 8% and 12% by weight, while for the diesel oil removal efficiency, thePC1 was influenced by water contents equal to 8% and 15% by weight;

3. on the score plot, the points of the microcosms were far from the other when their parameters were at the highest value used for the experimental runs.

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Carbon sequestration by horticultural plants in East Calcutta Wetlands ecosystem, a Ramsar Site in India

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Abstract

East Calcutta Wetlands (ECW) located at the eastern fringe of Kolkata metropolitan, India designated as a Ramsar Site (No. 1208). ECW receives municipal solid wastes and composite wastewater from the city and local artisans use this for horticulture, agriculture and pisciculture practices. Horticulture designated as 'green industry' sequester significant amount of carbon (C) in plant biomass and in soil, therefore, helps to mitigate greenhouse gases and combat against global climate change. Six horticultural plant species commonly cultivated in ECW ecosystems were selected for present study. C sequestration potential of horticultural plants depends on plant biomass and plant density in cultivation fields. Highest amount of C was sequestered (40.76 ± 6.73 ton ha^{-1}) by ixora, a perennial plant in plant biomass. In case of basil, also a perennial plant, significant portion of the living plant biomass was exported from ECW as economically important parts which constitute 5.79 ± 0.96 ton ha^{-1} C. Highest amount of soil organic carbon (SOC) was recorded from marigold fields (50.36 ± 24.88 ton ha^{-1}) which also constitute highest amount of C (34.37 ± 5.67 ton ha^{-1}) in residual parts (RP). Horticulture practices can sequester more amount of C in the ecosystem either by residual parts or remaining living plant biomass than agricultural practices.

Keywords: Carbon sequestration, crop residues, East Calcutta Wetlands, horticulture, soil organic carbon

Introduction

East Calcutta Wetlands (ECW) is located at the eastern fringe of Kolkata metropolitan city and designated as a Ramsar Site (No. 1208) for the ecosystem goods and services it provides. It receives different forms of solid wastes and wastewater (both municipal and industrial) from Kolkata and ameliorates it by natural processes (Chattopadhyay et al. 2002). Pisciculture, agriculture and horticulture are common practices in which local peoples of ECW area are engaged. Garbage farming, use of sludge from wastewater carrying canal in agricultural fields; use of wastewater in pisciculture ponds and irrigating agro-horticultural fields are traditional practices that are performed by local artisans. It is recorded that the solid waste dumping area, located at the western part of ECW ecosystem was converted to horticulture since 1876 (Kundu 2010).

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Horticulture, designated as 'green industry' is rapidly growing agricultural sector in rural, suburban and urban landscapes. Common horticultural practices include cultivation of ornamental or flowering plants, medicinal plants, aromatic plants, fruit orchards etc. These economically important products are exported and earned a livelihood for a number of people. Though horticultural practices may have some negative impacts on the global climate (Nicese and Lazzerini 2013), it also plays a crucial role to reduce greenhouse gases (GHGs) emission and hence increase carbon (C) sequestration potential (Marble et al. 2011, Ganeshamurthy et al. 2020).

Soil is an important abiotic component which store and sequester C and designated as the largest terrestrial pool of sequestered C (Lal 2003, 2004 and 2013). Soil organic carbon (SOC) is affected by land use, land cover changes and management practices (Manna et al. 2013, Scharlemann et al. 2014). SOC sequestration is the simplest and most cost-effective opinion to combat against global warming over the short-time horizon of 30 to 50 years (Govers et al. 2013, Paustian et al. 2019). Agriculture and agroforestry ecosystems create C sinks and are well studied in terms of SOC sequestration potential (Schahczenski and Hill 2009, Kay et al. 2019), though very sparse studies are conducted in horticultural fields (Marble et al. 2011, Alvarez et al. 2018). However, horticultural practices have a greater potentiality to sequester C in the terrestrial ecosystem than agroforestry or agricultural systems (Ganeshamurthy et al. 2020). Besides SOC pool another potential C sink in the horticulture sector is sequestered C in plant biomass. Living plant biomass is important C stock. Plant photosynthesis is responsible for large-scale C capture and storage (CCS) from the atmosphere.

Besides wastewater irrigated agriculture and wastewater-fed pisciculture, horticulture is also a year-old practice in ECW area. C sequestration potential of agricultural plants in ECW was studied by Pal et al. (2016a). C sequestration in different abiotic factors (Pal et al. 2018), trophic levels of pisciculture ponds (Pal et al. 2016b) and wetland macrophytes (Pal et al. 2017) were also well documented. However, the C sequestration potential of floriculture or horticulture practices from this area is still not assessed. The previous study highlighted the role of floriculture in the uptake of waste metals from soil and clean up the environment by phytoremediation (Chatterjee et al. 2012). Chatterjee et al. (2012) studied the role of three common plant species (viz., sunflower, marigold and cock's comb) commonly used in floriculture in ECW in bioremediation process. The present study aimed to find out the C sequestration potentials of selected horticultural plant species in living biomass and also in soil of ECW ecosystems.

Material and Methods

Study site:

Plant and soil samples were collected from Chowbaga (22.5317° N, 88.4128° E) located within ECW ecosystem. Among the six horticultural plant species that were commonly cultivated in ECW ecosystem, only two species were perennial and rest were annual (Table 1). Basil (*Ocimumtenuiflorum*), an aromatic subshrub, was a medicinal plant and it was also used in

different religious purposes or in rituals. Mainly leaves (along with flower) of basil were exported. Whereas in case of theshrub species,ixora (*Ixorachinensis*), flowers were used for decoration. Rest of the species, viz., Sunflower (*Helianthus annuus*), marigold (*Tagetes spp.*), cock's comb (*Celosieae spp.*) and globe amaranth (*Gomphrenaglobosa*) were annual herbaceous and were cultivated for flowers. Economically important plant parts were marketed (MP) and the rest of the biomass remains left behind in field either as residual parts (RP) in soil (in case of annual plants) or in living plant biomass (LB, in case of perennial plants).

Table 1. Selected horticultural plant species cultivated in East Calcutta Wetlands ecosystems.

Name of the plants	Family	Nature	Economic parts
Sunflower (*Helianthus annuus*)	Asteraceae	Annual herbaceous	Flower
Ixora (*Ixorachinensis*)	Rubiaceae	Perennial shrub	Flower
Basil (*Ocimumtenuiflorum*)	Lamiaceae	Perennial, aromatic subshrub	Leaves (along with flower)
Cock's comb (*Celosieae spp.*)	Amaranthaceae	Annual herbaceous	Flower
Marigold (*Tagetesspp.*)	Asteraceae	Annual herbaceous	Flower
Globe amaranth (*Gomphrenaglobosa*)	Amaranthaceae	Annual herbaceous	Flower

Sample collection:

20 plant samples of each plant were randomly collected to determine the average C content in different plant parts like root, stem, leaf and flower.Surface (0-10 cm) soil samples were collected randomly from each horticultural field by a handheld core sampler (having a diameter of 5 cm). Surface soils were collected as they were generally associated with root rhizosphere and were influenced by the root activity (Gregory 2006). Soil samples were collected randomly from each horticulture field in triplicate. Samples were collected on vernal months, viz., January to March 2018 between 08:00 AM to 10:00 AM. After sample collection, they were brought in the laboratory for further analyses.

Carbon content estimation in plants and soils

Wet weights (ww) of the samples (both plants and soils) were taken using electronic balance (Mettler Toledo AE 240 analytical balance).Then the samples were dried in hot-air oven at 60 °C until constant weight was attained. Dry weights (dw) of the samples were also recorded. Samples were grinded and homogenized into three separate aliquots for replication. 2.000 ± 0.01 mg of dried samples was weighed in tin foil using Perkin-Elmer AD6 Autobalance controller from each aliquot. These samples were then dropped into a high heat oxygen environment of combustion chamber in CHN analyzer (Perkin-Elmer CHNS/O, series II, 2400) for analysis of carbon. For each plant sample C content was estimated by following equation:

Total C content in a plant = (DB of root × CR) + (DB of stem × CS) + (DB of leaf × CL) + (DB of flower × CF)

Where, DB is dry biomass (g); CR, CS, CL and CF are C content in root, stem, leaf and flower respectively (g g^{-1})

For estimating the number of plants per hectare (ha) area 5 quadrats (of 10 m^2) were randomly laid from each horticulture fields. Each quadrat was surveyed, and the number of plants were counted. Mean value for each species from randomly chosen plots were considered for the estimation ofC stock in anarea (ha) by multiplying total C content in a plant and numberof plants in ha area.

Physicochemical properties of soil:

For the estimation of bulk density i.e., BD (Miller and Donahue 1990), moisture (Plaster 2008) and porosity (Matko 2003) both wet and dry weights were considered for necessary calculations. Sediment texture analysis was carried out following Wentworth (1922) and Gee and Or (2002) and as per their recommended methodology soil samples were fractioned into sand (<63 μm) and silt-clay (>63 μm).Soil pH and salinity (in terms of electrical conductivity i.e., EC) were estimated following Kandeler and Gerber (1988) and Bado et al. (2016) respectively. The pH and EC were determined by pH-Conductivity meter (WTW Multi 3420).

Statistical analyses:

The regression equations based on total dry biomass (TDB) and C content in different parts of six horticultural plants were generated using Microsoft Excel datasheet (Office 365). Dendrograms were constructed using hierarchical cluster analysis to group the studied plants according to C sequestration potential in living biomass of plants and in soil (SOC). Hierarchical cluster analysis was made based on Euclidean distances and single linkage method.Post-hoc measures (Tukey's HSD) were performed to highlight significant differences between the C sequestration potential ofsix selected horticultural plants based on plant biomass and in soil (SOC). Statistical analyses were carried out using Past version 4.01 software.

Results

Carbon sequestration potential in plant biomass varied widely (Table 2). Highest amount of C was sequestered by ixora (40.76±6.73 ton ha^{-1}) whereas lowest value was recorded for globe amaranth (7.13±1.19 ton ha^{-1}). In case of basil significant portion of the living plant biomass was exported (i.e., MP, 5.79±0.96 ton ha^{-1}). Lowest amount of C was exported from ixora (0.55±0.09 ton ha^{-1}). For annual plants like sunflower, cock's comb, marigold and globe amaranth significant amount of C was left behind in field as residual parts (RP). This amount was varied from 5.77±0.97 ton ha^{-1} (in globe amaranth) to 34.37±5.67 ton ha^{-1} (in marigold). Residual parts subsequently decomposed as soil organic matter. In case of perennial plants (like ixora and basil) leftover portion of plants after marketing of the economic parts existed as living biomass (LP) which can further grow. From Table 1 it can be estimated that the selected six plants on average contain 26.03±2.55ton ha^{-1}C in living biomass. Among this 9.68 % i.e. 2.52±0.38ton ha^{-1}C was exported from ECW.

Table 2. Total amount of carbon (C) exported through economical parts (MP) and present within the residual parts (RP) in soil or living plant biomass (LB) of selected plant species and soil organic carbon (SOC) from each field.

Plants	Amount of C in MP (exported) (ton ha^{-1})	Amount of C in RP in soil (ton ha^{-1})	Amount of C in LP (ton ha^{-1})	Soil Organic Carbon (SOC) (ton ha^{-1})
Sunflower	1.84±0.43	25.96±6.01	—	43.85±16.89
Ixora	0.55±0.09	—	40.21±6.64	36.10±3.88
Basil	5.79±0.96	—	22.13±4.10	37.54±3.88
Cock's comb	2.62±0.60	12.60±2.91	—	34.08±7.50
Marigold	2.94±0.49	34.37±5.67	—	50.36±24.88
Globe amaranth	1.36±0.22	5.77±0.97	—	42.45±9.79

SOC storage potential also varied among horticultural fields (Table 2).Highest amount of C storage was recorded from marigold field soils (50.36±24.88 ton ha^{-1}) whereas lowest amount was recorded from cock's comb (34.08±7.50 ton ha^{-1}). Soils from perennial plant fields also contain lower amount of SOC. Different physical and chemical properties of soil like BD, moisture, porosity, sand and silt-clay content, pH and EC were also varied widely among studied horticultural fields (Table 3).

Table 3. Physicochemical properties of soil from different horticultural fields of ECW ecosystem.

Soil samples	Bulk density (g cm^{-3})	Moisture (%)	Porosity (%)	Sand (%)	Silt and Clay (%)	pH	EC (µS cm^{-1})
Sunflower	0.85±0.12	16.49±7.58	13.47±5.08	55.86±9.29	44.14±9.29	7.28±0.06	102.27±21.17
Ixora	0.69±0.04	16.25±7.58	11.10±4.91	69.65±4.27	30.35±4.27	6.85±0.09	109.35±12.88
Basil	0.75±0.03	25.32±6.25	18.99±4.34	74.52±0.56	25.48±0.56	7.13±0.03	127.01±23.84
Cock's comb	0.70±0.18	21.27±18.44	13.77±10.87	60.98±3.79	39.02±3.79	7.14±0.09	94.57±15.96
Marigold	0.71±0.39	19.93±15.97	9.46±7.41	51.56±6.70	48.14±6.70	7.14±0.14	128.13±17.53
Globe amaranth	0.64±0.16	16.55±4.31	7.88±4.49	48.36±4.52	51.64±4.52	7.08±0.12	93.77±20.52

Significant differences (at $p< 0.05$) among carbon sequestration potential by studied horticultural plants revealed by Tukey'sHSD for pairwise comparison is depicted in Table 4. Significant differences were observed among sunflower-ixora, sunflower-cock's comb, sunflower-globe amaranth, ixora-basil, ixora-cock's comb, ixora-globe amaranth, basil-cock's comb, basil-globe amaranth, cock's comb-marigold and marigold-globe amaranth. However, there were no significant differences between SOC storage potential of different horticultural plants (Table 5).

Table 6 represents the relationships between total dry biomass (TDB) and carbon content in different plant parts of horticultural plants. All the plant parts viz., root, stem, leaf and flower

Table 4: Pairwise comparison values obtained by ANOVA (Tukey's HSD) within six horticultural pants based on C sequestration potential in plant biomass (* marked values with bold faces are significant at $p < 0.05$).

	Sunflower	Ixora	Basil	Cock's comb	Marigold
Ixora	0.02543*				
Basil	1	0.02705*			
Cock's comb	0.03122*	0.0000231*	0.02936*		
Marigold	0.1536	0.9321	0.1618	0.0001442*	
Globe amaranth	0.0003167*	0.0000004839*	0.0002977*	0.2886	0.00000234*

Table 5. Pairwise comparison values obtained by ANOVA (Tukey's HSD) within six horticultural pants based on SOC sequestration potential.

	Sunflower	Ixora	Basil	Cock's comb	Marigold
Ixora	0.9129				
Basil	0.9615	1			
Cock's comb	0.8033	0.9998	0.9974		
Marigold	0.9564	0.4701	0.5792	0.3329	
Globe amaranth	1	0.9605	0.9869	0.884	0.9062

Table 6. Relationships between total dry biomass (TDB) and carbon content in different plant parts of horticultural plants in ECW.

Plant parts	Regression equation	Correlation coefficient (R^2)
Root	y=2.281x+3.569	0.997
Stem	y=2.244x+15.828	0.996
Leaf	y=1.174x-13.640	0.983
Flower	y=2.251x-0.089	0.980

followed a similar pattern and the regression equations are of straight line with correlation coefficient (R^2) values ≥ 9.80.

Results of hierarchical cluster analyses based on sequestered C content in plant biomass and SOC are portrayed by dendrograms (Fig. 1). Dendrogram constructed based on plant C content showed two distinct clusters, one cluster was made up of cock's comb and globe amaranth and the second one was represented by rest of the four plant species. However, dendrogram constructed based on SOC depicted a different result. In this dendrogram marigold appear as an outgroup whereas rest of the plants formed a single cluster. Ixora and basil were appeared as most closely associated sub-cluster.

Discussion

Carbon sequestration potential of horticultural plants varied widely and depends on plant biomass. Higher biomass was achieved either by higher individual plant weight or by a higher

Figure 1. Hierarchical cluster analyses based on C sequestration potential in plant biomass (A) and soil (B) of six studied horticultural plants from ECW ecosystem.
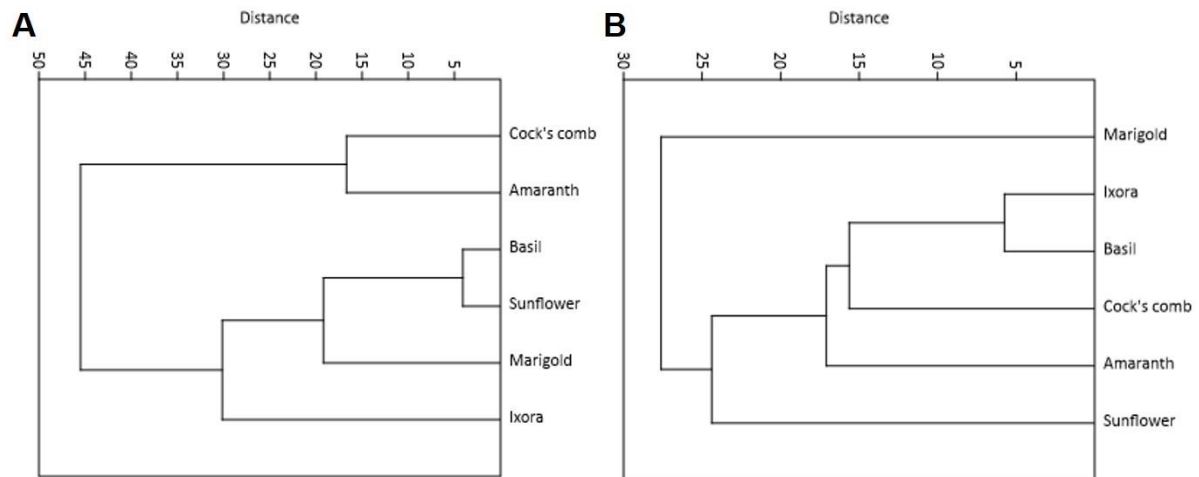
Figure 1. Hierarchical cluster analyses based on C sequestration potential in plant biomass (A) and soil (B) of six studied horticultural plants from ECW ecosystem.

density of plants per unit area. Both marigold and ixora contain a higher amount of C in plant biomass than the rest of the species. Ixora was a perennial woody shrub,which had the highest weight for an individual plant sample. However, in case of marigold individual plant weight was much lower but the number of plants per unit area was greater. Though the situation was different in case of these two plants but were responsible for the same result in terms of C storage in plant biomass. This result was reflected by hierarchical cluster analysis where these two plant species formed a cluster while dendrogram was constructed based on C content in plats.

Horticultural plants were cultivated mainly for ornamental flower except for basil which is cultivated for its leaves. Economically important plant parts were exported and the remaining portion of the plants was allowed to grow further. For annual herbaceous plants, after the growing season is over most of the biomass portion i.e., leaf, stem and root became a part of the soil organic matter. Crop residues were responsible for increased organic matter content in agricultural field soils i.e., they were an important source of C in soil and had long residence time (Singh and Rengel 2007). In the present study, marigold contented the highest amount of C in RP. Horticultural field of marigold was also possessed the highest concentration of SOC. Marigold appeared as an outgroup in dendrogram constructed based on the SOC values. Therefore, from the present study, it can be assumed that the higher the residual part biomass higher the input of organic carbon in the soil. Like agricultural fields, RP also played important role in enhancing SOC content in horticultural fields.

Pal et al. (2016) recorded that the C sequestration potential of eight agricultural plant species cultivated in ECW ecosystem was 6343.7 kg ha-1 C among which 4030 kg ha-1 C was

exported and 2313.6 kg ha-1 C remained in the field as residual parts. It means that about 63.53 % of sequestered plant C content was exported as economic agricultural products. In the present study, it was recorded that much less amount of sequestered plant C was exported from

horticultural fields. Therefore, horticulture practices can sequester more amount of carbon in the ecosystem either by residual parts or remaining living plant biomass.

India had 46.67 Mha area of wasteland among which 39.24 Mha areas were marginal lands (NRSC-ISRO 2011). A substantial portion of the area can be brought under horticulture or plantations of biofuel crops etc. (Edrisi and Abhilash 2016, Ganeshamurthy et al. 2020). Sustainable utilization of lands through horticulture also improve soil productivity, soil diversity and prevent soil erosion. Horticulture practices of profitable commercially important plants not only support the local economy and livelihood of farmers and other stakeholders but also enhance C sequestration potential of soil. Horticulture is an emerging land use pattern in rural, suburban or urban environments and has both positive and negative impacts on climate change like other ecosystems like agroecosystem, wetlands etc. Sustainable management practices can make horticulture sector more effective in reducing atmospheric GHGs emission by sequestering C (Marble et al. 2011). The potentials of horticultural fields to sequester moreC than agricultural fields will provide an opportunity for local farmers to credit in global C trading market.

Acknowledgements

Conflict of interests

Authors have no conflict of interests.

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Soap formation in biodiesel production: effect of water content on saponification reaction

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Abstract

In biodiesel production, soap formation creates phase separation problem during purification process resulting in low the biodiesel yield and ester content. This research evaluates the effect of water contents (0.05-1 wt%) on the saponification reaction of refined palm oil (RPO). In comparison, saponification of fatty acid methyl ester (FAME) with varying water contents (0.05-1wt%) are determined. From a microscopic point view, soap should act as a mass-diffusion barrier affecting a lower reactant mass flux to the reaction zone. Regarding soap formation in biodiesel production, the high-water content leads to soap formation in FAME and RPO due to hydrolysis, neutralization and saponification reaction. This work suggests that the soap formation behaviour in biodiesel production should be addressed; and thus, the optimal water content in raw material oils should be determined.

Keywords: Biodiesel, Hydrolysis reaction, Soap formation, Saponification reaction, Water content

Introduction

Biodiesel is defined as a mixture of alkyl esters which produced from the reaction between vegetable oils, animal fats, or waste oils containing triglyceride (TG) as the main component and short-chain alcohols (typically methanol or ethanol) in the presence of a suitable catalyst [1]–[3]. There are widely production methods; such as esterification, transesterification, and two-step method (esterification and transesterification). The reaction is called transesterificationreactionas shown in Eq. (1).

$$TG + 3\ Alcohol \underset{}{\overset{Catalyst}{\longleftrightarrow}} 3\ Ester + Glycerol \qquad\qquad Eq.\ (1)$$
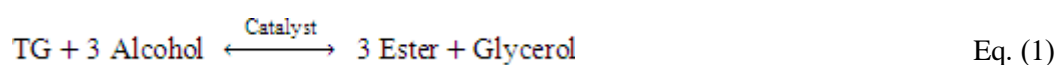
In the reaction, there are two types of catalysts which are a heterogeneous)solid(and a homogeneous)liquid(catalyst. The solid catalyst, either acid or base catalyst, is advantaged for low soap formation, but it is relatively time-consuming in the reaction. It required a high amount of alcoholand consequent separation equipment[1]–[3]. On the contrary, the liquid catalyst such as alkaline hydroxide and alkaline methoxide iswidely used in commercial biodiesel plants. It consumes less time, low alcohol usage, and is well mixed in the reaction. However, it could facilitate the saponification reaction and requires a high amount of water in a latter washing process[4]–[8].The soap formation is also the main reason for the biodiesel yield loss]3-4[. For commercial target, the low-cost feedstocks such as crude palm oil and waste cooking oil are interesting for biodiesel production. However, the presence of free fatty acids)FFA(and moisture contents in these oils lead to a serious problem of soap formation]4-5[.
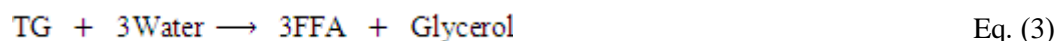
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They can speed up the hydrolysis of triglycerides (Eq. 3) and esters (Eq. 4)by increasing FFA to further react with the alkaline catalyst to form soap]1,5-7[. The chemical reactions of soap formation in biodiesel production are shown in Eq. (2-6). It is formed by FFA neutralization)Eq. 2)]1,5[and saponification of triglyceride) Eq. 5)[11]and FAME) Eq. 6([11] during transesterification in the presence of a homogeneous base catalyst. The saponification reaction is highly undesirable because it not only consumes the catalyst but also causes a problem in phase separation and emulsion formation during purification stages]3,5-7[. Hence, it reduces biodiesel yield. Therefore, the FFA content in the feedstocks is essential to render saponification during the transesterification process] 4,7[. Some studies have demonstrated the influence of FFA on transesterification using an alkaline catalyst to determine the optimal conditions] 1,7,8,9[. Kwiecien et al [11]demonstrated that ester content in the glycerol phase increases with increasing of soap content caused by the FFA neutralization and presence of water.
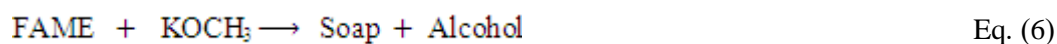
The free fatty acid (FFA) neutralization reactions are described in Eq. (2).

$$FFA \;+\; KOCH_3 \longrightarrow Soap \;+\; Alcohol \qquad \text{Eq. (2)}$$
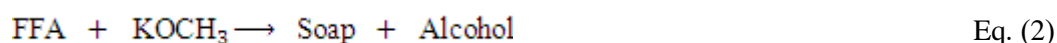
The hydrolysis reactions of triglyceride and ester are described in Eq. (3-4).

$$TG \;+\; 3Water \longrightarrow 3FFA \;+\; Glycerol \qquad \text{Eq. (3)}$$

$$Ester \;+\; Water \longrightarrow FFA \;+\; Alcohol \qquad \text{Eq. (4)}$$

The saponification reactions of triglyceride and ester and the potassium methoxide are described in Eq. (5-6).

$$TG \;+\; 3KOCH_3 \longrightarrow Soap + Alcohol \qquad \text{Eq. (5)}$$

$$FAME \;+\; KOCH_3 \longrightarrow Soap + Alcohol \qquad \text{Eq. (6)}$$

The key parameters affecting the yield of biodiesel in biodiesel production are FFA and moisture contents. According to industrial biodiesel companies such as Lurgi GmbH[13]and Crown Iron Works[14], these have specified feedstock properties as maximum acidity 0.1% or 0.5% and maximum moisture and volatiles as 0.1% or 0.05% . These impurities are significant to soap formation in the transesterification process. Among the otherreaction parameters, FFA content, catalyst type,and its concentration, and reaction time play key roles in biodiesel yield, which is related to soap formation is summarized in Table 1. The reaction kinetics are significant in biodiesel production. The immiscibility of alcohol and triglyceride causes a mass-transfer barrier during the transesterification process [15]. For a two-phase reaction, mass transfer with a chemical reaction and two-film theory are well described by Levenspiel[16]. Slinn (2008) proposed a mass transfer limited model adapted from Levenspiel (1999): the immiscibility of oil and methanol causes rigid glycerol droplets to form at the interface of methanol and triglyceride[17]. Tubino et al. (2014, 2016) proposed that methanolysis with alkaline catalysts should be heterogeneous[18].Noureddini and Zhu (1997) proposed a reaction mechanism consisting of an initial mass transfer controlled region followed by a kinetically controlled region [19].

Table 1. The effect of FFA content, catalyst concentration, and reaction time in biodiesel production.

Feedstocks	Transesterification conditions					Ester content	References
	FFA (wt%)	Catalyst (wt%)	Methanol/oil molar ratio	Time (min)	Temp. (°C)	(wt%)	
Used frying oil (UFO)	0.0-4.0	KOH (0.8-1.5)	27:1	30	30-50	>96.5	[10]
Waste cooking oil (WCO)	1.5-5.5	NaOCH$_3$(0.6-3.0)	18:1	5	60	>96.5	[20]
Refined palm oil (RPO)	0.2	NaOCH$_3$ (0.8)	6:1	30	50	97.05	[21]
Refined palm oil (RPO)	0.25	KOCH$_3$ (1.1)	6:1	30	50	97.31	[22]

Our previous works studied the actual events in liquid-liquid mass transfer transesterification via microscopic visual observations at room temperature [21]–[23]. The miscibility of biodiesel (FAME) and refined palm oil (RPO) from homogeneous alkaline catalysis (KOCH$_3$) via mass transfer by diffusion was studied on a concave glass slide as a micro-reactor. The study of soap formation behavior was included in the microscopic observations. Convective mass transfer in alkaline-catalyzed transesterification was evaluated.This study aims to evaluate the effect of water content on soap formation during the transesterificationreaction of refined palm oil (RPO) and the saponification reaction of fatty acid methyl ester (FAME). This study also aims at illustrating the reaction zone and the microscopic visualized observation of saponification reaction of saponification reaction using an LCD digital microscope.

2. Materials and Method
Chemicals and materials

Refined palm oil (RPO) with approx. 0.1 wt% of FFA and 0.1 wt% ofmoisture content, and a commercial-grade FAME (approx. 98.0 wt% purity, 0.1 wt% FFA) were obtained from the Specialized R&D Center for Alternative Energy from Palm Oil and Oil Crops, Prince of Songkla University, Thailand. Acommercial-grade methanol (MeOH, 99.8 wt% purity) was purchased from P-General Co. Ltd. Commercial grade potassium methoxide (KOCH$_3$, 32 wt% in methanol) was bought from AGC Chemicals (Thailand) Co., Ltd.The RPO and FAME were premixed with water for 0.05 to 1.1wt% water. All raw materials, RPO and FAMEwere analyzed the moisture content by a Karl Fisher coulometer.

Experimental methods
Study of the effect of water content on saponification reaction in biodiesel production

Three replicated experiments were conducted in a 1-liter 3-necked flat-bottomed flask equipped with a condenser and a 500-rpm magnetic stirrer. The reaction conditions of saponification were: 100g of refined palm oil (RPO) with varying FFA approx. 0.05-1.1 wt%. The RPO (0.1 wt% FFA) was premixed with water for 0.05 to 1.1 wt% water. KOCH$_3$ concentration is 0.0915 mol/L (2.54 g KOCH$_3$ + 135.21 g methanol). A sample of RPO was poured into the glass reactor, heated to 50 °C of reaction temperature followed by the potassium methoxide solution. The mixture was then stirred for

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the next 10 min, and approx. 3 ml of three replicated samples were collected using a volumetric pipette at 10 min. Three replicated samples were transferred into the pre-weighed 250 ml Erlenmeyer flasks containing 50 ml of isopropanol to immediately halt the reaction, and then analyzed for catalyst and soap contents. For biodiesel (FAME), the same procedure was carried out.

Study of the reaction zone and microscopic visualized observation of saponification reaction in biodiesel production

The microscopic observation of saponification reaction on a microreactor at room temperature (about 27 °C) was illustrated to determine the zone of reaction. A concave glass slide was used as a microreactor. This trial was performed by microscopic observation (Novel NLCD-307, 2.0 Mega Pixels CMOS Chip, at 100X magnification) of the mass diffusion and chemical reaction between the alkaline-methanol and triglyceride phases. A small pool of RPO (approx. 10 μL) at room temperature was put on the concave glass slide and centeredunder the LCD 307 microscope (Figure 1). A very fine drop of methanol-potassium methoxide -phenolphthalein solution (1 μL) was dropped in the pool of RPO. The concentration of potassium methoxide-methanol is 0.0915 mol/L.For microscopic visualized observation studies, three replicated samples (approx. 0.5 μL) were put on the concave glass slide and centered under the LCD 307 microscope.These trials were repeated several times. For biodiesel (FAME), the same procedure was performed.

Figure 1. An LCD digital microscope (Novel NLCD-307)

2.3 Analytical methods
2.3.1 FFA content analysis

FFA contents in oil and biodiesel were evaluated using an acid-base titration method (AOAC 940.28). Approx. 10 g of sample was transferred into a pre-weighed 250 ml Erlenmeyer flask containing 50 ml of isopropanoland then titrated with 0.05-0.1 N sodium hydroxide (NaOH) solutions using phenolphthalein indicator.

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Catalyst and soap contents analysis

The halted sample has tested the contents of catalyst and soap (AOCS Cc 17-79). The sample was analyzed by a two-step titration method with 0.05-0.1 N hydrochloric acid solutions: using phenolphthalein indicator for the catalyst content while bromophenol blue was used for the soap content.

Results and discussion

Study of the effect of water content on saponification reaction in biodiesel production

In alkaline-catalyzed transesterification, depletion of catalyst activity is due to its consumption by side reactions: neutralization and saponification. To determine the influence of water contenton soap formation, experiments were conducted by varying the water content of palm oil and biodiesel (FAME) between 0.05and 1.1 wt% as presented in Figures 2 and 3.

An increase of the water content in RPO and biodiesel (FAME) increases the high soap formation by hydrolysis and neutralization reactions (Figure 2 and 3). As a result, it was observed that the soap amount was higher than the total mole of FFA and water. The results suggest that soap was possibly produced via different sources; neutralization of FFA)Eq. 2)and saponification of triglyceride)Eq. 5(or FAME)Eq. 6(. The FFA content in raw materials leads to soap formation [11]. In comparison, biodiesel ease to form soap than palm oil due to the lower mass transfer barrier. From our previous studies [21], high FFA content in RPO creates a large portion of soap that restrains the diffusion of alcohol and catalyst. Formation of soap should reduce the remaining catalyst for further transesterification and thus the lower ester content would be obtained.Therefore, 0.1 and 0.05wt% are at the maximum tolerable water content in oil for transesterification reported by Lurgi GmbH[13]and by Crown Iron Works[24], respectively. The thick boundary layer of soap does not allow the diffusion of alcohol and catalyst to the reaction zone and has the consequence of slowing down transesterification.
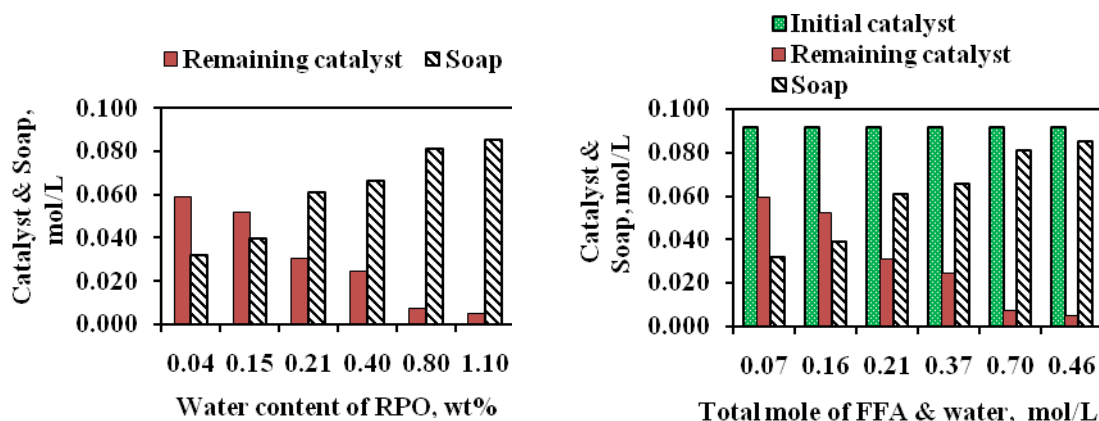

Figure 2. The effect of water content of RPO on saponification reaction in biodiesel production: the varying FFA content in RPO)0.05-1.1wt%(, FFA content)0.1 wt%(, total FFA and water)0.07-0.46 mol/L(, the potassium methoxide-methanol solution(0.0915 mol/L(, the reaction time of 10 min, 50°C and 500 rpm.

Study of the reaction zone and microscopic visualized observation of saponification reaction in biodiesel production

In this study, the transesterification of oil (Eq. 1) and saponification of triglyceride (Eq. 5) are expected reactions. For soap formation of biodiesel (FAME), it could be reacted with an alkaline substance which is expected from three major reactions, hydrolysis (Eq. 3 and 4), neutralization (Eq. 2) and saponification of FAME (Eq. 6). From Figure 4A, saponification of alkaline solution on RPO is expected to behave differently from FAME due to the transesterification reaction [21], [22]. Transesterification of triglyceride gives ester and as by-product glycerol. Glycerol is a strongly polar compound, unlike other esters. Glycerol should prefer to stay with methanol, and if the reaction takes place near the interface of alcohol and triglyceride, the glycerol should leave the ester and move to the methanol phase. Figure 4B, a drop of FAME on the pool of alkaline-methanol solution, indicates diffusion of FAME into the alkaline-methanol solution [21].
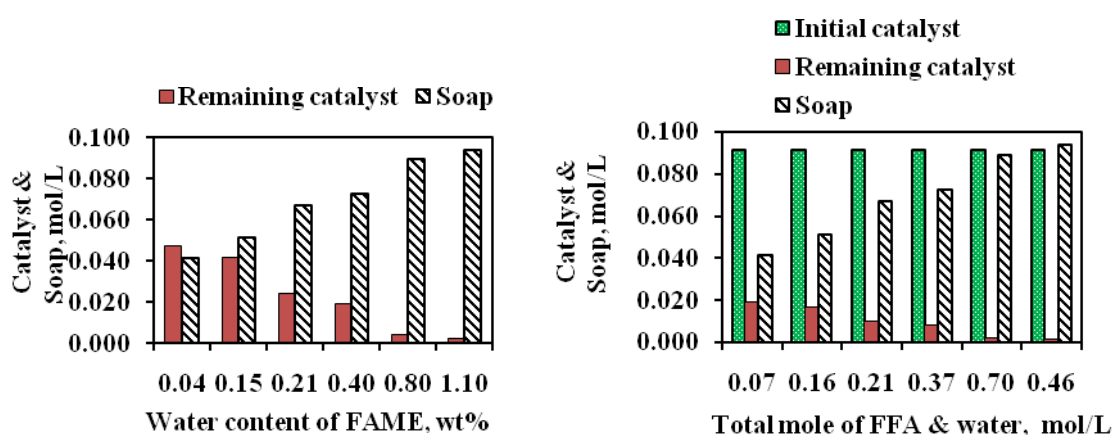

Figure 3. The effect of water content of FAME on saponification reaction in biodiesel production: the varying water content in FAME)0.05-1.1 wt%(, FFA content)0.1 wt%(, total FFA and water)0.07-0.46 mol/L(, the potassium methoxide-methanol solution(0.0915 mol/L(, the reaction time of 10 min, 50°C and 500 rpm.
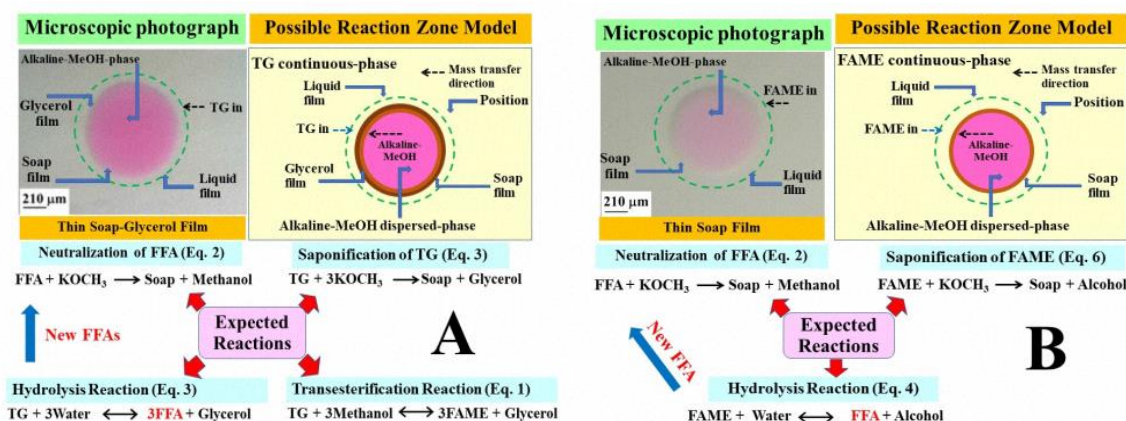

Figure 4. Possible reaction zone (40X magnification) and expected reactions: saponification of triglyceride and FAME (modified from Chanakaewsomboon et al 2019 [21])

The microscopic visualized observation (100 X magnification) of saponification reactions of RPO and FAME are presented in Figure 5. The results are clearly seen that pink droplets of the remaining potassium methoxide-methanol-phenolthalein. The two phases of alcohol and oils (RPO and FAME) from saponification are observed. The pink color indicates alkaline-phenolphthalein-methanol drops separated from the miscible methanol-FAME solution orthe miscible methanol-RPO solution [23].
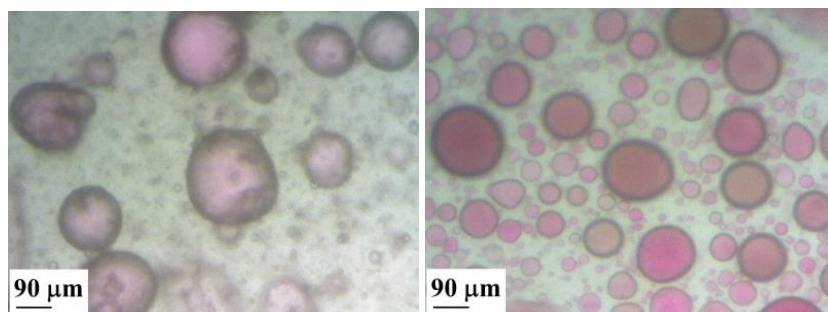
RPO   **FAME**

Figure 5. Microscopic visualized observation (100X magnification) for saponification of triglyceride and FAME.

Conclusions

In transesterification via alkaline catalysis, soap formation is a major factor of catalyst depletion and yield loss due to saponification reaction and loss on purification step. The rate of saponification reaction is a complicated solution that depends on many factors such as free fatty acid (FFA)content, water content, alkaline category, catalyst amount, reaction temperature, amount of methanol, and amount of glycerol, and many others factors. This paper demonstrates the effect of water contents of palm oil and biodiesel are performed in range 0.05 to 1.1 wt%. Microscopic observation investigates the actual events in liquid-liquid mass transfer transesterification at room temperature. Soap could act as a mass-diffusion barrier affecting a lower reactant mass flux to the reaction zone. High water content of oils (palm oil and biodiesel) leads to soap formation because of hydrolysis, neutralization and saponification reactions. Thus, the optimal water content in oil should be addressed.

Acknowledgements

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A brief commentary on activated carbon material as an efficient low-cost material for water decontamination

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Abstract

Activated carbon material, the low lost but very effective absorbent, can easily be synthesized from various naturally occurring bio-waste plants containing the enriched carbonaceous materials, like,coconut shell, peels of various fruits and vegetables, agricultural byproduct, husk, flower, carbonized lignite, coal etc.The high adsorption efficiency of such materials is the resultant of the high degree of porous structure and numerous reports are available on their properties. Development of such natural adsorbents attracted many researchers due to its specific efficiency for removing inorganic and organic compounds other than heavy metals from waste water. Unlike various other photocatalytic nanoparticles, the fundamental advantage of using activated carbon to remove pollutants from water is that it does not produce toxic byproducts after adsorption. However, substantial efforts on improving the potential activity of carbon are still on the run using varying chemical treatment and surface modification to improve its enhancement towards specific contaminants. This report primarily focuses on the raw precursor's material, the route of synthesis, different methods of modification and factors influencing the adsorption potential of activated carbon. Detailed information on the improvement on the surface, properties and its application on the water treatment have also been provided. Research reports on removal of harmful dyes, heavy metal, and emerging pollutants from waste water, water remediation are taken into account.

Contents:

1.Introduction

2.Preparatory methods of Activated carbon

 Physical activation

 Chemical activation

 3.Removal of Pollutants using Activated carbon

 Heavy metals

 Emerging pollutants

 Dyes

4.Conclusion

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Introduction

As far as 3750 B.C, wood charcoal was used for smelting and combining metals to create bronze by the Ancient Egyptians. Also, in between 1500-400 B.C, Ancient Egyptians and Hindus made use of wood charcoal as an adsorption material for medicinal purposes, purifying agent, and water purification [1]. Later in the late eighteen centuries, two chemist Scheele and Lowitz discovered adsorptive property of Activated carbon (AC) by decolorizing toxic gases in liquid phase which consequently revolutionized the sugar industry using AC as a decolorizing agent. However, the usage of activated carbon potentially was maximized during the first world as gas mask against toxic gases [2–4]. Hence, powdered and granulated activated carbon has found application not only during the first world but also used as an excellent adsorbent material till today.

AC also known as carbonaceous material or black carbon consists mainly of well-developed inner pore structure, large porosity and substantial surface area. Apart from it, the large porosity, high surface, well developed pore structure consists of three tunable pore structure such as micropores (<2nm), mesopores (2-5 nm) and macro pores (5-50 nm). Additionally, the production of AC can materialize from various naturally occurring bio-waste plants containing the enriched carbonaceous materials such as coconut shell, peels of various fruits and vegetables, agricultural byproduct, husk, flower and thecarbonized lignite and coal [5]This biomass waste can be converted into useful and low cost absorbent material through pyrolysis with or without the activating agents[6].

Generally, AC can be prepared from precursor carbonaceous material through physical activation (CO_2, air, steam), chemical activation (KOH, $ZnCl_2$, H_3PO_4), hydrothermal, microwave or a combination of the above methods. It has been investigated that the properties of AC solely depend on the precursor and are governed by the types of carbonization temperature, activating agent, time and impregnation ratio[7].Also the presence of different functional group on the surface of AC makes it multifaceted materials which have myriad application of research areasnotably in the environmental sector.Nonetheless, the presence of functional group(especially oxygen containing functional group) considerably influences the adsorption properties of AC. These functional groups are mainly derived from the activation process, precursor's material, chemical treatment.

As such, the functional group can be further modified by suitable chemical treatment in order to enhance its efficiency for specific water contaminant removal[8].

The high adsorption efficiency of ACs as adsorbent materials for different types of pollutants has been reported by many researchers. Unlike various photocatalytic nanoparticles, the fundamental advantage of using AC to remove pollutants from water is that it does not produce toxic byproducts after adsorption. Using agricultural biomass as a precursor for AC has created much attention towards many researchers due to its specific efficiency for removing inorganic and organic compounds other than heavy metals from waste water[9–12]. However, substantial efforts on improving the potential activity of carbon are still on the run using varying chemical treatment and surface modification to improve its enhancement towards specific contaminants. Though there are numerousmethods which have been reported in the literature on the surface modification of AC which throws a limelight not only on the uptake of specific contaminants on the surface of AC but rather gives information about the chemical and physical characteristics before and after modification[2,13,14]. However,factors such as temperature, adsorbent dosage, pH, contact time etc.also predominantly influences the adsorption efficiency of AC[15,16].

Hence, this review primarily focuses on the raw precursor's material, the route of synthesis, different methods of modification and factors influencing the adsorption potential of AC. Also, the adsorption capacity of different pollutants onto AC synthesized either by physical, chemical impregnation and through other modification has been presented. Furthermore, some information on the improvement on the surface, properties of AC and its application on the water treatment have also been well-furnished.Additionally, varying pollutant removal such as dyes, heavy metal, and emerging pollutants onto ACs are reported in this literature.The significant of this study is to provide lucidinformation and knowledge on the low cost carbon, effectiveness of AC as an adsorbent material towards the decontamination of various pollutants. Hence, this elucidation can serve as an insight out information on the potential application of biomass-

derived AC as substituted material in comparison to that of the expensive available methods for water remediation.
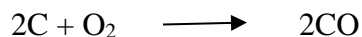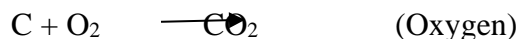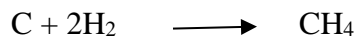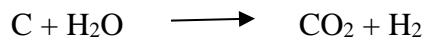
Preparatory methods of Activated carbon

Till today, commercially available ACs is still used for treating wastewater by variousindustrial sectors due to the pollution guidelines by government agencies. This commercial available ACs is largely produced from coal, coconut shell, bamboo, corncob, rice husk, wood etc[17–20]. The most important aspect of using ACs is its regeneration ability unlike other membrane technology and photocatalytic nanoparticles. However, most industries are on the run for low cost carbon derived from biomass due to its potential efficiency, porosity andenhanced surface functionality [11,12,21,22].

Since last decade, a larger number of researches on the efficiency of low cost biomass ACs for various pollutants have been conducted. Generally, the synthesis of biomass ACs proceed with the pre-treatment of the precursor material, drying, grounding and sieving to obtain uniform sizes. After obtaining the specific size range, it is then pyrolyzed at 200-700℃ in the presence or absence of inert atmosphere to obtain the desirable carbonized material or char[11,12,15,16,23,24]. After carbonization of the biowaste, the carbonized material is required for further activation using the conventional methods (physical & chemical)to enhance its surface chemistry, porosity and remove other impurity products left during the carbonization process. Regardless to the conventional methods, some other activation methods such as microwave, hydrothermal and magnetic modificationhave also been investigated [18,25–31].

Physical activation

Physical activation commonly is a two-step process. Firstly, the precursor's material is pyrolyzed to obtain the carbonized material. Then the carbonized material is activated with the involvement of oxidizing gases at an elevated temperature (500- 1000℃)[32,33]. The gases mainly comprise of carbon dioxide, steam, air or mixture of these gases. During the initial carbonization process, the pores formed during the process can be blocked by the presence of tar or other impurities. The presence of such tar or impurities can lower the adsorption efficiency of the carbon material [34–36]. Therefore, the idea towards gasification or activation on the carbonized material is to remove any blockage caused by the impurities or tar and also widen the porosity[37,38]. The chemical reaction occurring at such event with oxidizing gases is summarized as:

$$C + CO_2 \longrightarrow 2CO \qquad \text{(Carbon dioxide)}$$

$$C + H_2O \longrightarrow CO + H_2 \qquad \text{(Steam)}$$

$$C + H_2O \longrightarrow CO_2 + H_2$$

$$C + 2H_2 \longrightarrow CH_4$$

$$C + O_2 \longrightarrow CO_2 \qquad \text{(Oxygen)}$$

$$2C + O_2 \longrightarrow 2CO$$

Among the gases, carbon dioxide is the most frequently used gas for activation due to its slow reaction rate, safe and easy to handle even at higher temperature. It was suggested the ACs produced through physical activation do not possess greater surface or pore characteristics to use as an excellent adsorbent material.[39–42]. However, with the controlled condition and process parameters, Zaini et al and co-workers effectively synthesized activated carbon fiber with a surface area of 2938 m^2/g from phenolic fiber via steam activation at 900 °C for 1 h[43]. Zhang et al and co-workersstudied the effect of steam activation on the surface chemistry and pore structure of waste bamboo[44]. Their study reveals the surface area of the waste bamboo increased with increasing temperature with BET surface of 870 mg/g. It also suggested a decline in the surface area at low temperature could be possibly due to the formation of tar. Additionally, micropore volume decreases with the increasing activation temperature.

Chemical activation

In the chemical activation process, carbonization and impregnation using activating agent on the precursor can be carried out simultaneously in a single step at a temperature between 400-700 °C.However, two steps process i.e.firstly impregnation using suitable activating agents followed by further carbonization at desirable temperature range has also been applied[45–47]. The activating agents primarily comprises of acidic (H_3PO_4, H_2SO_4, HNO_3 etc.) and basic(KOH, NaOH, K_2CO_3 etc.) reagents[46,48–52].The advantage of using single step activation over two-step process of the precursor material is that it offers less time, low temperature and convenient porous structure. Though higher cost is involved during activation, chemical activation is highly preferred over physical activation due to higher yield, greater number of porosity,more efficient and lesser activation time[23,24,53]. In simplicity, chemical activation involves the incorporation of

activating agents to the carbonized material or char and heating with temperature range between 500-900℃.

In recent time, potassium hydroxide (KOH) and potassium carbonate (K_2CO_3) have been used widely in the manufacture of low cost ACs. Studies on the formation high micropore volume by KOH have been found and drawn much attention on its activating property when compared to ACs produced from H_3PO_4 and $ZnCl_2$ activation[54–58]. Besides, KOH also enhances the surface area of ACs[59], restricts tar formation [60]and lead to formation of functional group (O-H) on the carbon surfaces[61]. Li et al. reported the successful production of AC with high surface area of 2763 m^2/g at temperature of 750 ℃ from KOH activation with Kraft lignin as the precursor material[62]. Another study reported by Zhang et al.stated the KOH activation of hemp stem produced AC with high surface area of 2388 m^2/g[63]. In their study, hemp stem was firstly carbonized at 500℃ for 1h under N2 environment and then grounded. The grounded particles were soaked in 50% KOH concentration (impregnation ratio of 4.5:1) for 24h, dried and later the impregnated dried particles were activated at 800℃ for 1.5h. Similar studies has also been reported on the high surface area of 2696 m^2/g prepared from rice husk via KOH activation at 850℃ for 1h Muniandy et.al [64].

Removal of Pollutants using Activated carbon

Heavy metals

Contamination of ground and surface water by toxic metal has become an emerging concern over the last few decades due to their toxicity, persistence and bioaccumulative nature[65–69].Studies has shown the high contamination level in aquatic fish which can not only effect the species population but also can be subjected to other living organism which consume it[70–74]. Thus, efforts on its removal has been initiated with adsorption as one of the novel low-cost technique in day to day[75–77]. Rao et al. and coworkers successfully explored the potential application of activated carbon for Cu removal prepared from *Caryota urens* seeds[78]. Results from the experimental analyses gave valuable information on the effect of solution pH (1-10) dependency on Cu removal onto activated carbon. The uptake efficiency (%) of Cu(II) increased with

increasing solution pH with maximum uptake at pH range 4–6. Their study also suggests that at pH 1-2, the adsorption of Cu(II) was almost constant with removal efficiency of 30% attributing to the presence of high levels of H^+ ions. Also, the uptake efficiency of Cu(II) increased at pH 4 (95.7%) and attained a maximum removal efficiency at pH 6 (98.5%). However, the uptake efficiency of Cu(II) showed a decreasing trend when solution pH was increased to 10. The slight decrease in the adsorption of Cu(II) can be well-explained by the existence of Cu as an insoluble $Cu(OH)_2$ at pH>6 and the dominance of Cu^{2+} and $CuOH^+$ at pH ≤6. In addition, the effect of electrolyte solution (KNO_3) on the sorption uptake of Cu(II) was also investigated. It was concluded that the sorption of Cu^{2+} onto activated carbon decreased by the presence of electrolyte and could be potentially explained by the presence of K^+ ions which competes with the Cu^{2+} for the surface active sites.Dried coconut shell was converted into low cost adsorbent via physical activation and applied towards the removal of Cr(VI) from aqueous solution[79]. Herein, the effect of solution pH(2, 4) showed greater removal efficiency (94%, 86%) for uptake of metal ion on the activated carbon matrix which may attribute to the predominance of $HCrO_4^-$ with relatively smaller size in acidic solution (pH < 4.0) as compared to $Cr_4O_{13}^{2-}$, $Cr_3O_{10}^{2-}$, $Cr_2O_7^{2-}$ and CrO_4^{2--}. The greater removal efficiency at pH≤4 can be attributed due to the increased H+ ions on the modified adsorbent surface, creating the adsorbent surface more positively charged, thereby increasing the electrostatic attractions with negatively charged chromium ions. However, the sorption of the metal ion decreased with increasing solution pH>5 due to the increased negative surface charge on the adsorbent surface making it less feasible for interaction with negatively charged Cr(VI) ions. Similar study for removal of hexavalent chromium was selectively applied from carbon adsorbent prepared from apple peels[80]. The adsorption capacity of adsorbate-adsorbent solution decreases with increasing pH. It was concluded that at lower pH range of 1.0-6.0, $HCrO_4^-$ ions predominates as compared to $Cr_4O_{13}^{2-}$, $Cr_2O_7^{2-}$ and $Cr_3O_{10}^{2-}$.This can be explained by the increased protonation of H^+ ions making the adsorbent surface highly positive and subsequently favoring the electrostatic attraction with the negatively charged chromium ions. The unfavorable decline in the adsorption capacity with increasing pH can be attributed to the decrease positive charge on the adsorbent surface leading to electrostatic repulsion with the chromium ions in solution. From the experimental data, the sorption of Cr(VI) ions also suggested the Pseudo- 2nd-

order kinetics and Freundlich isotherm model better described the sorption process with maximum uptake value of 36.01 mg/g. As such for the other toxic metal, Vilvanathan and Shanthakumar et al. explored the removal efficiency from petals of *Chrysanthemum indicum* against the remediation of Ni(II) from aqueous solution[81]. In their study, pH played a pivotal role in affecting the degree of speciation, ionization and enhancement of surface charge of the biosorbent. It was observed that increasing pH significantly increased the adsorption uptake of adsorbate. This behavior can be attributed as with the increasing pH, the OH^- ions in solution increases, thereby enhances the negative surface charge of the biosorbent. However, at low pH value, the sorption uptake decreased due to the increase H^+ ion concertation. Subsequently, the H^+ ion occupy most of the surface active sites and leads to poorer sorption of Ni(II) via the electrostatic repulsive forces. Further, sorption kinetic and isotherm data provided was well-define by the pseudo-second order kinetic and Langmuir isotherm model suggesting chemisorption better describes the mechanism of adsorption process and sorption of Ni(II) as a monolayer on the surface of biosorbent. Similar reports on Ni(II) remediation was successfully explored using biomass of *Vigna unguiculata* pods[82]. It was observed that sorption of Ni(II) ions at pH 2 showed least uptake efficiency (8.74%). This may be attributed due to electrostatic repulsive forces between H^+ and Ni^{2+} ions and subsequent competition between the two ions for the active sites on the adsorbent. It also reported that uptake efficiency increased with increasing pH ranging from 2-5.5 (8.74 to 95.84%). However, at pH>5.5, the removal efficiency decreases due to the formation of soluble complexes of Ni(II) ions as (Ni(OH)2). Further, sorption kinetic and isotherm data provided best-fitted the pseudo-second order kinetic and Freundlich isotherm model with q_{max} value of 27.701 mg/g.

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Dyes

Among many other pollutants, textiles dyes are major contributor to water pollution which significantly affects the biological oxygen demand and pose threat to aquatic species.Hence, their removal from the aquatic system becomes an integral part for the environment. Studies on the removal of textile dyes (cationic and anionic) using biomass derived activated carbon has shown

remarkable achievement as low-cost sustainable adsorbent. Aljeboree et al and coworkers successfully explored the potential use of activated carbon from biomass-derived from coconut shell towards the removal of maxilon blue and direct yellow dyes (GRL and DY 12)[83]. In their study, maximum sorption uptake for both dyes was achieved at the lower pH (pH~3) and the minimum sorption at high pH value of 10. Additionally, the effect of particle size greatly influences the adsorption process indicating better uptake for smaller size particles enabling greater surface area. However, their study suggests less favorability in sorption uptake with the increasing temperature for GRL and DY12 onto the coconut shell activated carbon. Studies on the removal of reactive red dyes (RRD) using banana and orange peel via acid activation was explored by Temesgen et al and Coworkers[84]. In their study, the surface area for orange peel (336.224 m2/g) was found to be higher than that of banana peel. Based on the surface analysis, the experimental results showed higher capability for orange peel with both the adsorbent showing optimum pH of 4. The maximum adsorption percentage for reactive dyes at optimum pH was found at 89.41 % (orange peel) and 70.25% (banana peel). In addition, activated biochar prepared from *opuntica ficus-indica* was potentially utilized for the adsorption of malachite green (MG) from aqueous water.Studies from the Experimental investigation suggest the color reduction of maximum MG was achieved at pH 10 of 74% which could be explained by the structural changes in MG. Thus, the biomass derived material can be used as a low-cost and alternative material for treating textile wastewater more cost-effectively.

Emerging pollutants

Emerging pollutants (EPs) in the water bodies is a growing global concern. What exactly are these so called emerging pollutants? The United States Geological Survey (USGC) defineit as "any natural or synthetically occurring chemical or any microorganism which has the potential to enter the environment without being monitored and cause severe adverse effecttowards ecological balance, aquatic environment and also to human and animal life"[85–87]. The existence of EPs in ground or surface water is a major concern due to its potential effects on the total environment. Most of these EPs are released from drug industries, household products, municipal dumping sites,

human and animal excreta etc. These EPs mainly comprises oforganic and inorganic contaminants[88–90].

As such, pharmaceutical compounds or drugs are developed for the well-being of human and animal health. Some of these popular pharmaceutical drugs include antibiotics, painkillers, antidepressants, chemotherapy agents, non-steroidal anti-inflammatory acid drugs, tranquilizers etc[86,90–93]. However, most of these pharmaceutical compounds (PCs) when consumed, a considerable amount of it are not adsorbed. Such unavoidable conditions ultimately find its way to the environment via animal and human excreta and later into the sewage system or through leachate that seeps into the groundwater system. Mostly, the PCs are discharge into the water bodies by pharmaceutical industries, hospital effluents,municipal dumping sites and wastewater effluents. Unfortunately, most of the PCs are not biodegradable and are not completely removed during wastewater treatment plants. The presence of such pharmaceutically active compounds in the aquatic environment is a major threat due to its mutagenicity, high toxicity, carcinogenicity or development of resistant by bacteria. Consequently, they have been detected globally either in surface water and ground water or wastewater effluents more frequently. Based on the potential ill effects, numerous remediation techniques have been undertaken to remove EPs from the aquatic system[86–88,94–97]. Some of the commonly technique involves the electrochemical treatments , biological treatments , membrane filtration, advanced oxidation methods , adsorption nanofiltration, Ultrafiltration etc[98–104]. Nonetheless, among all the methods, adsorption methods have been of great advantages and considered the most promising techniques for removing EPs from the aquatic environment. This is mainly due its low-cost, regeneration ability, high adsorption efficiency and no toxic byproducts are formed after the treatment[105–107].

Non-steroidal anti-inflammatory acid drugs (NSAIDS) comprise of analgesics, anti-inflammatory, antipyretic pharmaceutical compounds. Some of the NSAIDS drugs such as ibuprofen (IBF), diclofenac (DCF), naproxen (NPX) and ketoprofen (KPF) have been widely detected in the aqueous environment (ref). Baccar et al. studied the adsorption of DCF, IBF, NPX and KPF prepared from agricultural biowaste derived from Olive stones by H_3PO_4 as an activating agent[108]. According to their studies, it was observed that the uptake efficiency of the four pharmaceutical drugs declines with increasing pH and the effect continued when the pH became

alkaline. It is also suggested that the π-π interaction between the aromatic ring of the activated carbon and the drug cannot substantially explain the decreasing adsorption trend with increasing pH.   Additionally, the Langmuir model best-fitted the experimental data with time-based investigations showed that sorption process was best explained the second-order kinetic model. Similarly, Mondal et al. and coworker's investigated the potential application of steam mung bean husk activated biochar to remove ibuprofen (IBP) from aqueous solutions[109].In their study, under optimized parametric conditions, the maximum removal of IBP was found to be 99.16%. Additionally, the equilibrium adsorption study reveals the Langmuir isotherm best explains the experiment data set with a maximum adsorption capacity of 62.5 mg/g. In another study, optimized removal of oxytetracycline (OTC) from contaminated waters using chemically activated and pyrolyzed biochars from forest and wood-processing residues was conducted by Aghababaeia et al. and coworkers [110]. Their study suggest the adsorption efficiency of OTC was enhanced substantially with increasing temperature from 20 to 40°C. Additionally, the chemical treated forest-derived biochar pretreated (5M H3PO4) showed increasing adsorption capacity from 14.7 to 95.1 mg/g within the temperature range between, 20 to 40°C. Further, studies showed decreasing trend in the uptake of OTC on the adsorbent due to the electrostatic repulsion between the negatively charged surface of adsorbent and the anionic form of OTC (OTC$^-$ and OTC $^{2-}$) at pH > 5.5.

Conclusion

The access to clean water is vital for human survival and the environment. The removal of contaminants from wastewater and water bodies using ACs has remarkably shown its removal ability and efficiency. ACs derived from agricultural waste and biowaste as an adsorbent has high potential compared to other existing materials. This is reasonably due to its low cost production, availability and better removal efficiency. Research studies on dye and heavy metal removal using AC had been largely investigated and considered to be a better material for removal by many researchers. But the exact mechanism of removal of such pollutants has to be well understood during multiple step activation. The adsorption studies of various pharmaceutical drugs have recently been of great interest. Though, there are no regulating permissible frameworks for

pharmaceutical drugs in the environment, the presence of it can be potentially hazardous to the ecosystem. The key advantages for removing pharmaceutical compounds using ACs is that it does not generate any toxicity or pharmaceutical byproducts as compared to other photocatalytic materials. Furthermore, the performance of pharmaceutical drugs and personal care products removal needs to be investigated in detail.

Through this brief review, the use of renewable agricultural waste for the production of activated carbon has been presented. It has been seen the myriad application and advantages of AC as an adsorbent for purification of various pollutants from natural water and wastewater. Though, there are multiple challenges to boost the production of AC. Efforts are definitely made to ensure to protect the environment from significant pollutants day after days due to the global demands.

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Cyanobacteria of the Indian Sundarbans: A Potential Source of Powerful Therapeutic Agents

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Abstract:

Mangrove forests occupy the estuarine ecotone and harbor a wide range of microorganisms along with a rich diversity of flora and fauna. Marine and estuarine organisms are known to produce unique molecules due to the aggressive, exigent, and competitive surroundings that are unlike those produced in the terrestrial environment. Marine cyanobacteria are a vast resource for new bioactive natural products useful in the development of therapeutics. The Sundarbans mangrove ecosystem harbours various unique microorganisms having different interesting properties. Discovery of a unique alkaline serine protease enzyme tolerant to bleach, detergent, high salt concentration and solvent, isolation and identification of obligatelyhalophilic,euryhaline novel cyanobacteria from intertidal soil surface of the Sundarbans and identification of a pair of novel Streptomyces represent a few of the ongoing endeavors undertaken to explore the mostly untapped microbial diversity of the Sundarbans. This study focuses on two novel strains of cyanobacteria isolated from the intertidal soil surface biofilm of the Indian Sundarbans, which were cultivated on a large scale to yield a significant quantity of biomass for the extraction of secondary metabolites. The cyanobacterial biomass was extracted with a range of polar and non-polar solvents and the ethyl acetate fractions showed significant anti- angiogenic activity when tested against sunitinib (a protein kinase inhibitor). The extracts also showed significantly greater anti- inflammatory activity compared to dexamethasone, which has been shown to reduce the 28 day mortality rate of patients affected by COVID- 19.

Keywords: Halophilic cyanobacteria, Indian Sundarbans, anti- inflammatory activity, anti- angiogenic activity.

Introduction

Modern day trends in drug discovery from natural sources stress the investigation of marine environment to yield numerous biologically active compounds many of which are antimicrobial in nature (Burja et al., 2001). The intertidal areasconsidered as interfaces of the ocean, atmosphere, and terrestrial environments harbour diverse microbial biofilm communities that are subjected to fluctuations in metal ion concentrations, temperature, desiccation, UV irradiation, and wave activities. The organisms in intertidal zones essentially spend part of their lives in extreme, arid conditions during emersion and half of their lives in stable, benign seawater. These conditions lead to the development of unique and specific characteristics of the inhabiting organisms (Zhang et al.,2013). These ecosystems experience tidal flooding, which causes environmental factors such as salinity and nutrient availability to be highly variable resulting in unique and specific characteristics of the inhabiting

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organisms. The microbiome is one of the important communities of the mangrove ecosystem as the abundance of carbon and other nutrients sustains a large number of microbial communities which are adapted to the brackish and fluctuating environmental conditions (Pramanik et al., 2011).Marine cyanobacteria are a vast resource for new bioactive natural products useful in basic research, biomedical sciences, and the development of therapeutics (Gogineni et al., 2018).

Natural products of pharmacological importance derived from cyanobacteria

Cyanobacterial natural products are grouped according to their biosynthetic origins such as polyketides, cyanopeptides, alkaloids, isoprenoids and other metabolites. While majorresearch has been towards investigating toxicity, many studies have shown cyanobacteria toproduce compounds that are of pharmaceutical and biotechnological interest. Cyanobacterialcompounds comprise 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolidesand 9% amides. Most of the bioactivity shown by cyanobacteria tends to be from lipopeptideslike cytotoxic (41%), antitumor (13%), antiviral (4%), antibiotics (12%) and the remaining18% include anti-malarial, antimycotics, multi-drug resistance reversing agents, herbicidesand immunosuppressive agents. A major part of cyanobacterial secondary metabolites arepeptides or possess peptidic structures. Many important classes of cancer cell toxins withapoptotic properties have been characterized from marine cyanobacteria over the past years.Effects of cyclic peptides as anticancer agent with multitude targets have been reviewed.*Lyngbyamajuscula*among other cyanobacterial genera collected from various coastal anddeep- sea regions of the marine environment worldwide has proved to be one of the mainsources for the production of natural products with anti-tumor and anti-cancer properties,regardless of their geographical distribution (Nagarajan et al., 2012). Many of the secondarymetabolites secreted by cyanobacteria were found active against different mammalian cancercell lines. Some important compounds isolated from cyanobacteria that target cancers aregiven as follows: colon cancers are targeted by minutissamides, microcystilide A,laxaphycins, cylindrocyclophanes and bauerines A-C while breast cancers are targeted bycarbamidocyclophanes, dendroamide, hapalosin and tolyporphins; lung cancer is are targetedby pahayokolide A; and prostate cancer by tychonamide. A few secondary metabolitesisolated from cyanobacteria were shown to have profound activity against certain parasitescausing deadly diseases. A compound isolated from freshwater cyanobacterium*Nostoc*displayed antiprotozoal activity against *Trypanosoma*and *Leishmania*and significant toxicityto malaria parasite. Pharmacologically important metabolites have been isolated from marinebenthic and planktonic cyanobacteria that inhibit growth of severe bacterial, fungal andprotozoal pathogens. Studying active concentration, active modules and mechanism of actionof bioproducts on both prokaryotic and eukaryotic pathogens and/or parasites will help intheir selection for clinical evaluation. A class of antifungal lipopeptides known aslobocyclamideswere isolated from marine cyanobacterium*L. confervoides*collected fromCay Lobos, Bahamas (Nagarajan et al., 2012; Raja et al., 2016). Freshwater and terrestrialcyanobacteria are also proven to have the potential to produce compounds displayingcytotoxic, protease inhibiting and growth controlling properties on parasites, pathogens andharmful algae. Further studies on these metabolites may make synthesis of drugs containingthe bioactive key components possible. Thus, a new vista may

open towards the treatment oflife-threatening diseases (Nagarajan et al., 2012). Despite the wide range of natural productsfound in cyanobacteria, exploitation of these products is still not widespread.

Studies on the cyanobacteria of the Indian Sundarbans

The Sundarbans mangrove ecosystem harbours various unique cyanobacteria having pharmacologically important properties. Isolation and subsequent identification of obligatelyhalophilic, euryhaline novel cyanobacteria from intertidal soil surface of the Sundarbans (Pramanik et al., 2011) represents one of theendeavors undertaken to explore the vastly unexplored microbial diversity of the Indian Sundarbans, under the guidance of Dr. J. Mukherjee (School of Environmental Studies, Jadavpur University). Based on morphological characteristics, six of the isolated cyanobacteria were assigned to the *Lyngbya-Phormidium-Plectonema* (LPP) group B, and one each was assigned to *Oscillatoria*and *Synechocystis* genera. A polyphasic approach- based taxonomic characterisation was performed for the cyanobacteria, which led to the discovery of four novel strains, out of which two are a novel species*O. aestuarii*belonging to the genus *Oxynema*. Cross walls in the apical portion of cells of the strains AP17 and AP24 were absentwhile the same were present in CCALA960. Additionally, optimal growth of AP17 and AP24 was recorded at 5–8% salinityand salinity above 14% inhibited growth of both strains, which were isolated from the intertidal soil surface; whereas *O. thaianum* CCALA960 which was found in a hypersaline environment could grow at 40% salinity. Differences between the internal transcribed spacer (ITS) sequences of the two strains isolated from the Indian Sundarbans and the reference strain included the insertion of 9 nucleotides in the D2 with spacer region, insertion of 2 nucleotides in the pre Box B spacer region, deletion of 2 nucleotides in the post Box B spacer region, deletion of 8 nucleotides in the D4 region, deletion of 8 nucleotides in V3 region and insertion of 2 nucleotides in the D5 region of the ITS sequences of AP17 and AP24, which were observed in comparison to the analogous regions of CCALA960. Structural details of Box B helices of AP17 and AP24 revealed that though their lengths were identical with that of the reference strain, their sequences were completely different from CCALA960. Four nucleotide substitutions were presentin different positions in the Box B helix of *O. thaianum* CCALA960. Secondary structures of the V3 regions of both AP17 and AP24 (containing 51 nucleotides) showed a small terminal bulge and a bigger bilateral bulge while the analogous structure of *O. thaianum* CCALA 960 (comprising of 59 nucleotides) showed one additional bilateral bulge in comparison to AP17 andAP24. Therefore, based on morphological, ecological and molecular differences in comparison to *O. thaianum* CCALA960, isolates AP17 and AP24 were proposed to be members of a second novel species in the *Oxynema* genus, for which the name *Oxynemaaestuarii* sp. nov.has been proposed (Chakraborty et al., 2018).The other two strains AP9F and AP25 are monophyletic taxa designated as *Euryhalinemamangrovii* and *Leptoelongatuslitoralis* (*gen. nov., sp. nov.*). The cells of AP9F and AP25 were highly elongated whereas the cells of the reference strains (*Leptolyngbyaboryana*and *Nodosilineanodulosa*) were occasionally elongated to isodiametrical. Terminal cells of AP9F and AP25 appeared as flattened corners (as opposed to rounded), which was different from the cell structure of other

Leptolyngbyaceae members. 16S rRNA gene sequences of AP9F (1366 bp) and AP25 (1408 bp) showed 95% and 92% similarities respectively with the non-redundant (nr) nucleotide sequences of their closest relatives of the *Leptolyngbya*genus. Test strains occupied a clade in the phylogenetic tree that was different from the ones containing the type species. A single operon containing both tRNA[ile] and tRNA[ala] genes were present in the ITS regions of AP9F and AP25 as compared to the presence of two operons in the ITS region of the reference genera *Leptolyngbya*and *Nodosilinea*: one in which both tRNA[ile] and tRNA[ala] genes are present and the other lacking both the genes. The secondary structures of the traditionally conservative D-stem region as well as the Box B helix and V3 regions of the ITS operons showed significant variation between the test strains and also when compared with the corresponding sequences of *L. boryana*and *N. nodulosa*. Molecular, phylogenetic and morphological data suggested AP9F and AP25 to be monophyletic taxa for which the names *Euryhalinemamangrovii gen. nov., sp. nov.*and*Leptoelongatuslitoralis gen. nov., sp. nov.*were proposed respectively (Chakraborty et al., 2019).Thus, the strains AP17, AP24, AP9F and AP25 isolated from the Sagar Island and Lothian Island of the Indian Sundarbans differed from the reference strains (*Oxynemathaianum* CCALA960 for *Oxynemaaestuarii*, *Leptolyngbyaboryana*, and *Nodosilineanodulosa*for *Euryhalinemamangrovii* and *Leptoelongatuslitoralis*) in terms of morphology, ecology and 16S- 23S ITS sequences (Chakraborty et al., 2018, Chakraborty et al., 2019). The aforementioned novel strains have been deposited and cryopreserved in the Microbial Culture Collection (MCC), India having accession numbers MCC 3874 (AP17), MCC 3873 (AP24), MCC 3171 (AP9F) and MCC 3170 (AP25).

Material and methods

Cyanobacterial isolates were established by aseptic collection of cyanobacterial soil surface biofilm, inoculation in artificial sea nutrient (ASN-III) medium, and subsequentincubation in fluorescent irradiance maintaining a 12-h:12-h light:dark cycle at $25\pm1°C$, and plating the seriallydiluted homogenized biomass obtained after 40 days of growth. Individual colonies of filamentous cyanobacteria were isolated after 30 days on ASN-III plates, observed microscopically, and grown in liquid ASN-III medium supplemented cycloheximide and triple-antibiotic solution (containing penicillin G, chloramphenicol, and streptomycin sulfate) to prevent culture contamination. Thecyanobacterial cell suspension so obtained was subsequently grown in antibiotic-free ASN-III medium for 30 days, and culture purity was confirmed by the absence of microbial growth intryptone-yeast extract-glucose (TYG) broth (Pramanik et al., 2011;Chakraborty et al., 2018). Mass cultivation of the established cyanobacterial monoculture strains was done by growing them individually in 20litre capacity plastic jars disinfected by washing withbenzalkonium chloride followedby addition of 12 litres of sterile ASN III media and ~5 gm (wet mass) of cyanobacterial culture added to each of the jars as inoculum. Aeration was achieved with the use of pumps to ensure proper mixing of the media components, along with maintenance of the light and temperature conditions for growth (Pramanik et al., 2011).The cyanobacterial biomass of each of the two strains (one belonging to *Oscillatoria* sp. and the other being *Oxynemaaestuarii*) thus obtained (~200g each) was dried at 50°C, divided into two parts and both parts extracted separately usingethyl acetate and n-butanol. The extracts were dried in vacuo and tested for anti- inflammatory activity and anti-angiogenic activity.The test for anti- inflammatory activity was performed using human

monocytic leukemia THP-1 cells that were pre-treated for 12h with standard compound Dexamethasone (1 μM). Subsequently, pretreated cells were stimulated with LPS- 50 ng/ml for 4h. After treatments, cell supernatant was collected for TNF measurement using ELISA. (According toClin Chem. 2005; 51(12):2252-6). Measurement of anti- angiogenic activity is based on the principle that the formation of capillary-like structuresamongendothelial cells plated at sub- confluent densities in matrigelmatrix in the presence of the compound under investigation extrapolates to angiogenesis (Goodwin, 2007).The cell line used for this *in- vitro* assay is theEA.hy926 endothelial cell lineobtained by the hybridization of human umbilical vein endothelial cells with the A549/8 human lung carcinoma cell line (Aranda et al., 2009).

Results and discussion

Secondary metabolites obtained from the cyanobacterial biomass extracts(with ethyl acetate) have shown promising anti- inflammatory activity and anti- angiogenic activity (the ability to prevent endothelial cells to form capillary-like structures) compared to the standards Dexamethasone and Sunitinib respectively (tested at CDRI, Lucknow) (unpublished report). Both the extracts from the cyanobacterial strains showed anti- angiogenic activity by reducing the capillary structures (>25% inhibition compared to standard compound, Sunitinib) at the initial 100 μg/ml test dose (Table 1). Both extracts also showed ≥75% inhibition of inflammatory activity compared to Dexamethasoneat the initial 100 μg/ml test dose (Table 2). Thus, the ethyl acetate extracts of these cyanobacterial strains can be further purified by column chromatography and HPLC for reduction of possible cytotoxicity.

Serial number	Extract Code	% Tubulogenesis inhibition
1	"Ethyl Acetate Extract" AP20	Active, lower dose to be tested
2	"Ethyl Acetate Extract" AP24	Active, lower dose to be tested
Standard	Sunitinib	~40

Table 1: Test results of ethyl acetate extracts of AP20 (*Oscillatoria* sp.) and AP24 (*Oxynema aestuarii*) for anti- angiogenic activity against Sunitinib (standard). Concentration of extract tested: 100μg/ml.

Source: Unpublished report, CDRI Lucknow.

Anti- inflammatory activity		
Serial number	Extract Code	% inhibition
1	"Ethyl Acetate Extract" AP20	≥85
2	"Ethyl Acetate Extract" AP24	≥85
Standard	Dexamethasone	~78

Table 2: Test results of ethyl acetate extracts of AP20 (*Oscillatoria* sp.) and AP24 (*Oxynema aestuarii*) for anti- inflammatory activity against Dexamethasone (standard). Concentration of extract tested: 100µg/ml.

Source: Unpublished report, CDRI Lucknow.

Conclusion

Despite the wide range of natural products of significant pharmacological value found in cyanobacteria, exploitation of these products is still not widespread. The growth ofcyanobacterial biomass is quite slow, which is certainly one of the most important limiting factors due to which massproduction of bioactive compounds is limited.Further studies on the metabolites isolated from the cyanobacterial biomass may help in the synthesis of drugs containing the key components contributing to the bioactivity possible (Nagarajan et al., 2012). The study may prove to be a beneficial step in the discovery of several compounds of pharmacological interest from the largely unexploredmangrove microbiota of the Indian Sundarbans, opening a new vista towards the treatment of life-threatening diseases.

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