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SYNTHESIS AND EVALUATION OF RICE STRAW LIGNIN BASED HYDROGELS AS CULTURE MEDIA FOR GROWTH OF POT MARIGOLD *(Calendula officinalis)*

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ABSTRACT

Rice straw is renewable, cost effective and abundantly available feedstock, which is usually burned on farm leading to emission of hazardous gases in North-West India. Therefore efficient utilisation of rice straw is important to reduce extensive atmospheric pollution. In the present study, rice straw was used to generate hydrogel which can be used as culture media for growth of flowering plants. The rice straw used in the study contained 20.00, 41.00, 24.00, 9.00 and 6.00 per cent composition of cellulose, hemicellulose, lignin and acid-insoluble ash (silica), respectively. Lignin from rice straw was extracted using the dilute acid pretreatment followed by its alkaline hydrolysis. Lignin was structurally characterized by FT-IR, ^{1}H and ^{13}C NMR spectra. Hydrogels were synthesized using lignin as raw material followed by their characterization using FT-IR and SEM analysis. Different concentrations of lignin hydrogels (0.2, 0.5, 0.8 and 1.0LH) were synthesized out of which 0.5LH proved to be the most efficient due to their high swelling ratio (130.34) and high reswelling capacity. Additionally, hydrogels (0.5 and 0.8 LH) served as great culture media for seed germination and growth of marigold, which showed greater root length, shoot length, root: shoot length ratio, seed vigour index, germination percentage and germination index. Therefore, it was concluded that 0.5 and 0.8 lignin hydrogels can be used efficiently as culture media to enhance the growth of flowering plant marigold.

Keywords: *Calendula*, Hydrogels, Lignin, Poly vinyl alcohol, Rice straw

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Rice straw, a widely available lignocellulosic biomass, is an attractive feedstock for energy and extraction of polymers for the synthesis of advanced functional materials (Isaeva *et al.*, 2024). Abundance of unavoidable rice straw and its open field burning is associated with greenhouse gas emission leading to environmental pollution (Hung *et al.,* 2020), which has led to recent advances in research for its utilization. Rice straw consists of 30-45% cellulose, 20-25% hemicellulose, 15-20% lignin and 4-6% minor organic groups (Hung *et al*., 2020). Lignin is the most abundant aromatic biopolymer and the third most abundant plant polymer in the world; and offers many advantages such as being biocompatible, non-toxic, eco-friendly, biodegradable, renewable, cost effective and easily available (Muhammad *et al.*, 2024). Therefore, lignin can be used for the production of value-added materials leading to a remarkable step forward, as it also contributes to the recycling management economy in terms of the biorefinery process.

In the past few years, lignin has been used in the adsorption of organic dyes (Silva *et al*., 2011), dispersants (Qin *et al*., 2015), plasticizers (Yu *et al.,* 2013), lignin-derived catalysts (Zhu *et al*., 2019), cancer therapy (Zhen *et al.,* 2020), antioxidant (Guilhen *et al*., 2017) and antimicrobial (Alzagameem *et al.,* 2019) agent. Recently, researchers have explored the formation of biopolymers-based materials such as hydrogels, biofilms, bioplastics etc. Lignin consists of an abundant amount of hydrophilic functional groups *i.e.* hydroxyl (20-30%), carbonyl (20%) and methoxyl (90-95%) (Zakzeski *et al.*, 2010) which act as reactive sites for chemical modifications. This makes lignin an excellent candidate for the production of bio hydrogels. Hydrogels are 3-D polymeric networks synthesized from cross-linked homopolymers, copolymers or macromers (Buwalda *et al.,* 2014). Hydrogels have gained much attention due to their property of high water retention within its matrix, upto a thousand times their dry weight (Hoffman, 2012). These hydrophilic gels (Bhattarai *et al*., 2010) have been in the spotlight of researchers for a while, as they have been employed in biomedical, agriculture and many other fields. For the purpose of formation of lignin hydrogels, water soluble, highly reactive polyvinyl alcohol (PVA) is generally incorporated into lignin forming chemically crosslinked lignin-PVA hydrogels (Li *et al*., 2018).

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PVA hydrogels have been widely synthesized as they have extensive applications in many fields like in drug delivery (Sun *et al*., 2012), biosensor (Yang *et al*., 2013) super-absorbents (Zhai *et al*., 2016) etc. However, PVA hydrogels lack biodegradability, have low recovery rate and brittle nature, which could be compensated by incorporation of biomass lignin resulting in improvement of these properties (Zhang *et al*., 2019). Lignin PVA hydrogels have been extensively used in wound dressings (Li *et al*., 2019), food packaging (Zhang *et al*., 2019) etc. These lignin hydrogels offer many applications in agricultural fields *i.e.* they can be used as culture media in the soilless germination of seeds in flowering plants. Recent scientific studies have shown the use of hydrogels as an effective method to improve the germination and growth of young coffee seedlings (Gokavi *et al.,* 2018). Hydrogels have also been used to improve soil moisture and yields of aerobic rice in hydrogel amended soil (Rehman *et al.,* 2011). Hydrogel improves water holding capacity, decreasing the irrigation requirements of several crops resulting in delay and onset of permanent wilting under excessive evaporation. Lignin derived hydrogel is highly effective in water absorption, thus its use may be explored as culture media for seed germination and growth of flowering plants to replace soil. Presently, limited information is available on the synthesis of lignin derived hydrogel from rice straw and its use as culture media for flowering plants. Soil-less growth of flowering plants is desirable for decorative purpose and also scientifically it's an easy way of controlled release of nutrients. Given this, the present study was planned to synthesize lignin hydrogels and evaluate their application as culture media in growing flowering plant *Calendula*.

MATERIALS AND METHODS

Straw of rice crop variety PB 1121 (Pusa Basmati 1121) was collected from Department of Soil Science, Punjab Agricultural University, Ludhiana, India. It was oven-dried at 60°C, ground, passed through 1 mm sieve and then stored in plastic bags in an air-tight container at room temperature. Pot marigold (*Calendula officinalis*) seeds were procured from the Department of Floriculture and Landscaping, Punjab Agricultural University, Ludhiana, India.

Determination of chemical composition of rice straw

Detergent based method was employed for the determination of major polymeric constituents

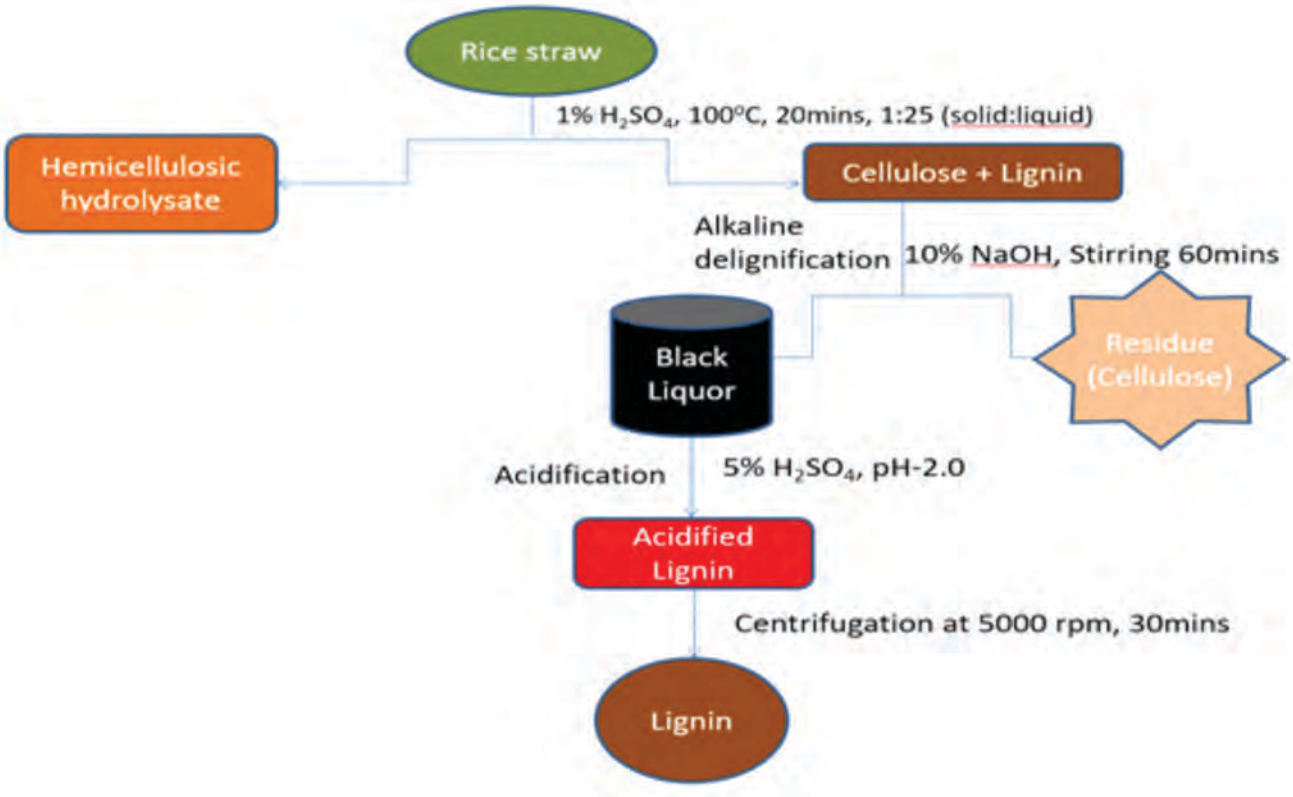


Fig. 1. Extraction of lignin from rice straw

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of rice straw *i.e.* cellulose, hemicellulose and lignin (Goering and van-Soest, 1970).

Extraction of lignin from rice straw

Rice straw sample was stirred (120 rpm, 20-25 min, 110°C) with 1% *(w/v)* of H_2SO_4 taking solid-to-liquid ratio of 1:25 (Fig. 1). The stirred mixture was cooled at room temperature and the solubilized fraction was processed with vacuum filtration. This was followed by alkaline hydrolysis using NaOH with 1:25 solid-to-liquid ratio followed by stirring and vacuum filtration forming a black liquid product. Further, the solution was acidified using 5% H_2SO_4 followed by centrifugation at 5000 rpm for 30 mins forming lignin precipitates. These precipitates were filtered and washed with distilled water (till pH 7) and dried at 70°C for 24hrs to obtain a dry lignin sample. The structural characterization of purified lignin was done by FT-IR, ^1H and ^{13}C NMR spectra.

Synthesis of lignin-PVA hydrogels

Four different lignin-PVA hydrogels, labelled 0.2LH, 0.5LH, 0.8LH and 1.0LH, were synthesized taking equal concentrations of both lignin and PVA *i.e.* 0.2, 0.5, 0.8 and 1g following procedure of Kaur *et al.* (2023). PVA was dissolved in 10ml of 2% NaOH solution in an oil bath at 90°C with continuous magnetic stirring at 500 rpm for 5 min. Lignin was added to PVA solution by stirring at room temperature to form a uniform solution. Finally, 0.20 mL of epichlorohydrin (ECH) was added and stirred again with heating at 75°C for 20 mins. Homogeneous gelatinous solutions of hydrogels were obtained which were immediately poured into the silicon moulds with a syringe and air bubbles were eliminated from the surface with poking. After this, hydrogels were washed thoroughly to eliminate non-reacted lignin and the residual NaOH. The hydrogels were then left at room temperature for drying. Lignin-PVA hydrogels were poured into moulds to take up the shape of small round balls and were allowed to dry.

Characterization of hydrogels

All hydrogels prepared in this study were analyzed by FTIR spectra recorded on FT-IR spectrophotometer (BRUKER Alpha -II**)** in the Electron Microscopy and Nanoscience laboratory at Punjab Agricultural University (PAU), Ludhiana, India. Scanning electron micrographs of hydrogels were scanned from central instrumentation laboratory, Division of Research and Development, Lovely Professional University, Jalandhar, Punjab, India.

Swelling and reswelling test

The weight of dry hydrogels was noted and then hydrogels were immersed in distilled water for 3 days. After that, swollen hydrogels were taken out and excess water was wiped out with filter paper and the weight of swollen hydrogels was noted.

The Swelling ratio (Q) was calculated using the following formula given by Wang *et al.* (2014):

$Q= W_s–W_d/ W_d$

where, Q is the swelling ratio, W_d is the total weight of the dry hydrogel in grams and W_s is the total weight of the swollen hydrogel in grams. This swelling ratio gives the amount of water contained per gram of hydrogel.

Lignin hydrogels were subjected to re-swelling tests in order to study the reusability of these hydrogels. The hydrogels were subjected to oven drying at 60°C and then were made to swell in distilled water for 3days. These cycles of drying (deswelling and swelling) were repeated 6 times and swelling capacities of these hydrogels were noted in each cycle. All hydrogels were taken in three replications for swelling and reswelling test.

Evaluation of hydrogels as soilless culture media for the growth of pot marigold

Pot marigold (*Calendula officinalis)* seeds (8 seeds/medium, 3 replications) were sown in beaker containing prepared hydrogels and kept in an incubator at 17 ± 2°C and 60% humidity under complete dark conditions till seedling emergence. Seeds sown in beaker contained soil was considered as control. Beakers were replicated thrice and arranged in completely randomized design in incubator. When the tip of the radicle had grown out of the seeds upto 1-2 cm, the seeds were considered germinated. Seedlings were plucked out from the hydrogel culture media and soil after 14 days of sowing and the following parameters were recorded from each replication.

Root length and shoot length (cm)

The root and shoot length were measured after washing and drying of the seedlings. The area below the hypocotyl to the root tips was considered as root length and the area between the hypocotyl and the terminal bud was considered as shoot length. Root to shoot length ratio was calculated.

Fresh and dry weight of seedling (g)

Fresh weight was immediately measured after

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Table 1. ^{1}H-NMR spectra of lignin

Sr. No.	Chemical shifts (δ, ppm)	Assignment
1	7.5	aromatic H of p-hydroxyphenyl units conjugated with a double bond; aromatic H of phenolic acids
2	6.69-7.0	aromatic-H in guaiacyl
3	6.26-6.29	aromatic-H in syringyl
4	3.32-3.71	H of methoxyl group
5	1.15- 1.23	aliphatic -OH
6	0.83-0.85	methyl in aliphatic saturated structure

plucking the seedling. Afterwards, the seedlings were dried in an oven at 70°C for 8 hours to measure the dry weight.

Germination index (GI)

The germination index was determined using following equation:

Germination index (GI) = ((G / G_o) * (L / L_o) * 100

where, G and L = number of germinated seeds and root length in hydrogel media, respectively;

G_o and L_o = total number of seeds sown and root length in the control conditions, respectively.

Seedling vigour index

The seedling vigour index was calculated by multiplying seedling length (cm) and germination percentage (Ambika *et al*., 2014).

Statistical analysis

The germination experiment was conducted in a completely randomized design using three replicates. The data were subjected to analysis of variance (ANOVA) using SPSS software (VR.25).

RESULTS AND DISCUSSION

Rice straw was found to constitute 20.0, 41.0, 24.0, 9.0 and 6.0% of extractives, cellulose, hemicellulose, lignin and acid-insoluble ash (silica), respectively. In FT-IR spectra of lignin, a strong band at 3420 cm^{-1} showed the presence of hydrogen bonded phenolic and alcoholic OH groups (Table 1). Presence of -CH_2 and -CH_3 groups in the structure of lignin was confirmed with a band which appeared at 2922 cm^{-1}. The stretching vibrations at 2882 cm^{-1} seemed to be characteristic of the -OCH_3 group. The bands of the aromatic group were observed at 1636, 1600, 1511 and 1424 cm^{-1} and the -CH deformation vibrations were observed at 979 and 897 cm^{-1} with aromatic -CH deformation at 1119 cm^{-1}. Further, the band for C-H bending of methyl groups was obtained at 1464 cm^{-1}. The bands at 1389 and 1334 cm^{-1} were associated with syringyl and guaiacyl rings, respectively, in lignin. Besides these bands, the strong bands at 1245 and 1040 cm^{-1} are characteristic of C-O stretching of aromatic and aliphatic esters while the band at 1165 cm^{-1} may be due to CO stretching of secondary alcohols (Li and Ge, 2018).

The signals obtained for lignin in ^{1}H NMR spectrum and the corresponding linkages (Wang and Chen, 2013; Lu *et al.,* 2017) are shown in Table 1. Further, the structural characterization of lignin

Table 2. Morphological parameters of pot marigold grown in soilless culture media (hydrogels) and control (soil)

Hydrogels	Root length (cm)	Shoot length (cm)	Root to shoot length ratio	Germination index (GI)	Seedling vigour index	Fresh weight of seedling (g)	Dry weight of seedling (g)
0.2LH*	0.95^{c} ± 0.13	1.06^{e} ± 0.2	0.92^{b} ± 0.27	5.01^{b} ± 1.34	32.92^{c} ± 11.61	0.54^{e} ±0.04	0.27^{d} ± 0.05
0.5LH	3.00^{a} ± 0.36	6.50^{a} ± 0.89	0.46^{b} ± 0.03	36.82^{a} ± 8.22	346.25^{a} ± 75.03	1.87^{a} ± 0.16	0.98^{a} ± 0.12
0.8LH	2.34^{b} ± 0.2	5.30^{b} ± 0.26	0.44^{b} ± 0.05	26.41^{a} ± 15.47	251.67^{ab} ± 140.48	1.28^{b} ± 0.09	0.61^{b} ± 0.06
1.0LH	0.83^{c} ± 0.06	0.13^{f} ± 0.06	6.83^{a} ± 2.02	5.85^{b} ± 2.19	20.42^{c} ± 8.32	0.27^{f} ± 0.02	0.14^{e} ± 0.04
Neat PVA	1.34^{c} ± 0.15	2.03^{d} ± 0.15	0.66^{b} ± 0.08	9.17^{b} ± 5.31	70.84^{c} ± 31.05	0.67^{d} ± 0.04	0.47^{c} ± 0.04
Soil (Control)	3.00^{a} ± 0.3	3.14^{c} ± 0.49	0.97^{b} ± 0.16	25^{ab} ± 21.65	148.75^{bc} ± 118.04	0.99^{c} ± 0.02	0.54^{bc} ± 0.02

Values are mean± standard error of three replicates
Within a column means followed by same alphabet do not differ significantly at $p<0.05$ after DMRT.
*LH: Lignin hydrogel

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was done with ^{13}C NMR spectra. The peak at 104.27 ppm belongs to syringyl rings whereas the signal corresponding to the methoxy group was shown at 55.79 ppm. The peak for the guaiacyl ring was observed at 115.54 ppm. Additionally, the peak at 152.46 accounted for either C_3, C_4 of esterified guaiacyl or C_3, C_5 of syringyl β-O-4 linkage (Saha *et al.,* 2018).

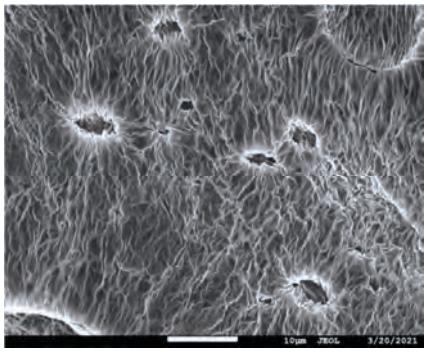
Dilute acid pretreatment was used to extract lignin from rice straw as it is cost effective and known to give accurate results (Hsu *et al*., 2010). It hydrolyses hemicellulose without changing lignin structure and acts by breaking ether linkages in lignin-carbohydrate complexes (Tarasov *et al*., 2018). The pretreated rice straw was subjected to alkali treatment for delignification as alkali disrupts lignocellulosic plant cell wall by causing swelling of cellulose, solubilizing lignin, hemicellulose and silica and by hydrolyzing acetic and uronic acid esters (Karuna *et al*., 2014).

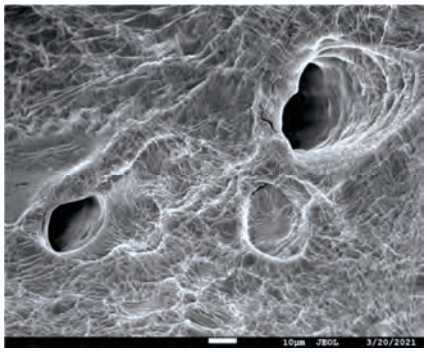
Characterization of hydrogels

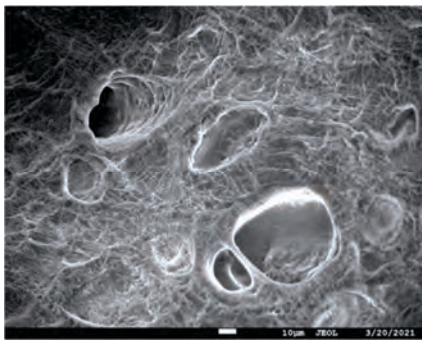
The FT-IR spectrum of lignin hydrogels showed a strong absorption band at 3334 cm^{-1} which is characteristic of O-H stretching vibrations due to presence of alcoholic and phenolic OH groups in lignin which are involved in hydrogen bonding. Lignin hydrogels showed an absorption band at 1639 cm^{-1} which belongs to aromatic skeletal vibration and a band at 1346 cm^{-1} due to carbon-carbon stretching vibrations in the aromatic ring. Weak bands in the region of 1180 cm^{-1} were also observed due to C-H in-plane bending.

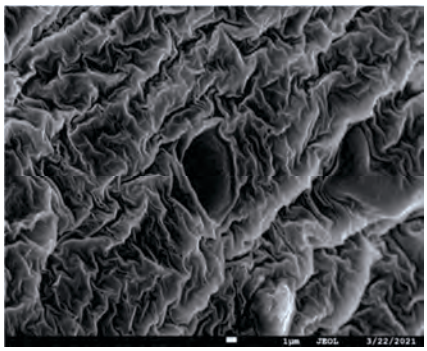
The SEM micrographs of 0.5LH revealed that it has the maximum pore size with a sponge like microstructure as compared to neat PVA hydrogels (Fig. 2B). This was followed by 0.8LH where the pore size was less than 0.5LH but more than all other hydrogels, confirming the presence of a good cross-linked network (Fig. 2C). Other hydrogels *i.e.* 0.2LH and 1.0 LH possessed a network of small pores which were scattered in the domain (Fig. 2A and D). SEM images of neat PVA hydrogels showed a more homogeneous distribution of pores throughout the network but the size of the pores was very small justifying its low water absorption capacity (Fig. 2E).

SEM analysis of all the hydrogels depicted that the average pore size of lignin hydrogels decreased with increasing lignin concentration. This may be attributed to the fact that high concentration of lignin leads to a large number of hydrogen bonds between OH groups of the lignin and PVA units


(A) 0.2LH


(B) 0.5 LH


(C) 0.8 LH


(D) 1.0 LH

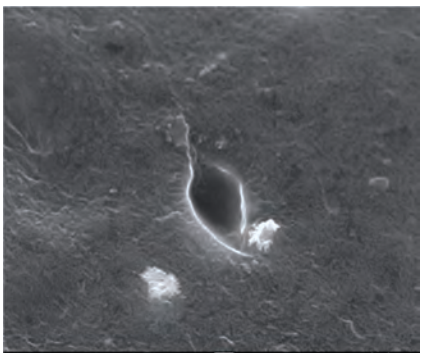

(E) Neat PVA hydrogel

Fig. 2. SEM micrographs of different hydrogels LH: Lignin hydrogel

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which leads to the formation of macropores (Ji *et al*., 2017). However, when lignin concentration is very high, then it may alter the crosslinking, thereby decreasing pore size. Lignin may act as a nucleating agent as it is believed to form macropores (Yang *et al*., 2018). Lignin nanoparticles are known to act as spacers and lead to a highly porous PVA/cellulose nanofibril structure (Bian *et al*., 2018). Lignin addition to PVA hydrogels led to the formation of thinner walls between the micro voids, which help to increase the water-retention ability of the hydrogels due to the higher contact surface with it and, thus, its diffusion. There was presence of open channels mixed with smoother interconnecting domains (Navarra *et al*., 2015) in 0.5LH. It was also observed that neat PVA hydrogels show a high degree of cross-linking which is due to massive hydrogen bonding (Wang and Gunasekaran, 2006) during the synthesis process.

Swelling ratio and re-swelling capacity

There was a significant increase in swelling ratio of lignin hydrogels as compared to neat PVA hydrogels. The swelling capacity of 0.5LH was the highest which was significantly (1.8 fold) more than neat PVA hydrogels. It was followed by 1.4 fold increased swelling ratio in 0.8, and 1.2 fold increased in both 0.2 and 1.0 LH than neat PVA hydrogel. The hydrogels with the highest amount of lignin (1.0 LH) had the lowest swelling capacity in all lignin hydrogels. After six cycles of deswelling and swelling, a slight decline in swelling capacity was observed after the fifth cycle. These hydrogels can be used 4-5 times with almost equivalent swelling capacity.

It is a well-known fact that lignin maintains a cross-linking network with PVA and when it is present in optimal concentration (Wu *et al*., 2019) and has high water absorption capacity. Swelling capacity is dependent mainly on initial lignin concentration and hydrogels washing stage where unreactive lignin gets washed off. If lignin is present in large amounts, then it will decrease the water absorption capacity of the hydrogels by altering the cross-linking structure. Lignin is added to PVA hydrogels as it enhances water absorption capacity by forming more pores. This was confirmed by lower swelling capacity (75%) of neat PVA hydrogels. This proved that lignin enhances the pore size and, therefore, the water retention inside the network of hydrogels. These lignin hydrogels were observed to possess the ability to perform well with good swelling capacity till 4 cycles of deswelling and swelling. Previous studies have claimed that PVA hydrogels lose swelling capacity when there is repeated swelling and deswelling (Wu *et al*., 2019) as repeated cycles of deswelling and swelling lead to the breakage of physical cross-linking within the hydrogels network. This breaking of the polymeric network leads to gradual loss in the ability to retain and absorb water at equilibrium. However, the samples in present work were still capable of absorbing considerable amounts of water.

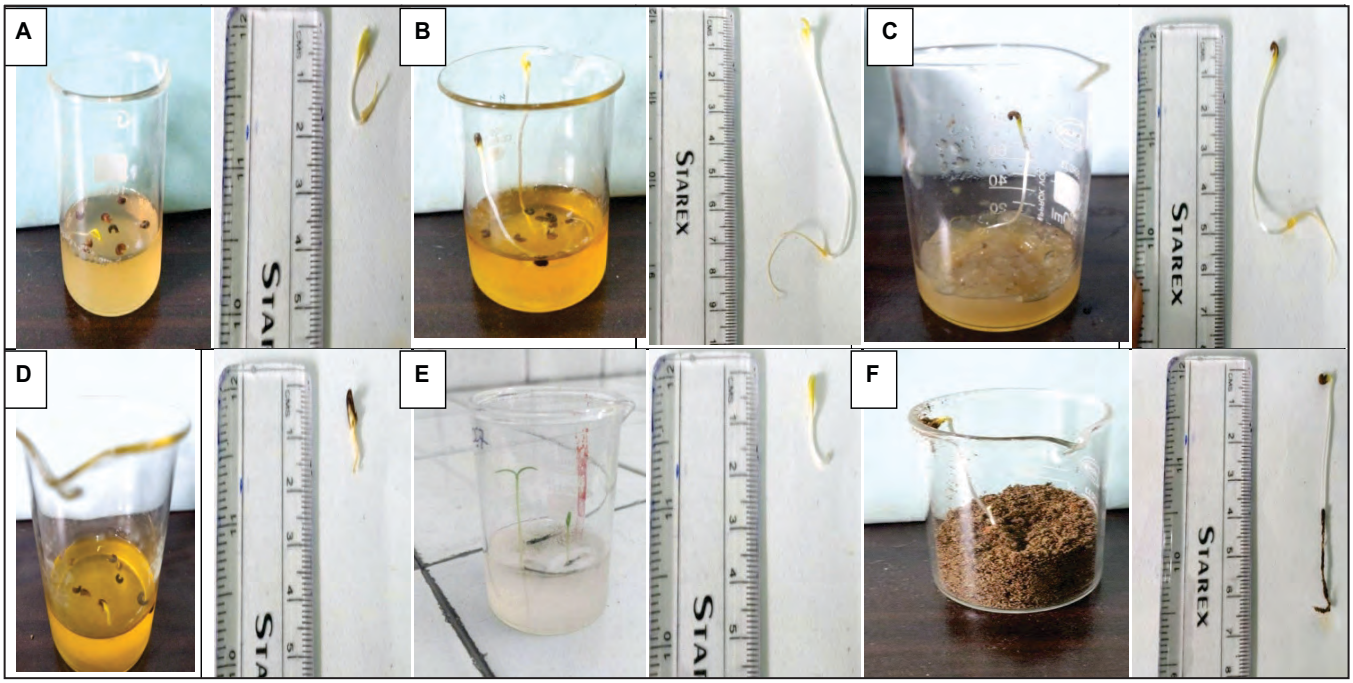


Fig. 3. Evaluation of hydrogels as culture media for growth of pot marigold
LH: Lignin hydrogel (A) 0.2LH (B) 0.5LH (C) 0.8LH (D) 1.0 LH (E) Neat PVA hydrogel (F) Soil (Control)

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Hydrogels as culture media for growth of pot marigold

There was a significant decrease in root length of *Calendula* seedlings in lignin hydrogels and neat PVA hydrogels except in 0.5LH (Table 2, Fig. 3). Root length showed no change in 0.5LH as compared to the control but increased in 0.2 and 1.0LH as they have less water absorbing ability. Hence, they provide lesser amounts of water to the seeds for their growth and the roots elongate to absorb more water.

Shoot length of *Calendula* significantly increased in 0.5LH and 0.8LH which was 2.07 and 1.69 fold more than control, respectively (Table 2, Fig. 5). Root to shoot length ratio decreased in all the hydrogels as compared to control. R: S ratio for 0.8LH was minimum in all the lignin-PVA hydrogels and control (Table 2, Fig. 3).

There was a significant increase in fresh weight of seedling in 0.5LH and 0.8LH by 1.88 and 1.29 fold, respectively, compared to control (Table 2) but a decrease in fresh weight for 0.2, 1.0LH and pure PVA hydrogel. Similarly, dry weight of seedling also increased significantly in 0.5 and 0.8LH by 1.81 and 1.13 fold, respectively as compared to the control.

Root length increased in 0.2 LH; because of the presence of a lesser amount of lignin in the hydrogels, crosslinking decreased due to absence of reactive sites of lignin (Wu *et al*., 2019). When lignin is present in higher amounts in hydrogels (1% LH), it causes abruption in the crosslinking structure (Huang *et al*., 2019) as epichlorohydrin (ECH) is also present in less amounts to be reactive with large amounts of lignin to form a crosslinked structure of lignin hydrogels. This shows that 0.5% LH is an optimum concentration for lignin to be efficient for seed germination as there was no change in root length in 0.5 LH. When the root to shoot length ratio is higher, it indicates that the root has high penetration in the soil which plays an important role in taking nutrients, water, agricultural production and stress tolerance. Under water deficient conditions, the root: shoot ratio increases to enhance soil exploration and nutrient uptake (Blaha, 2019).

Germination index (GI) and seedling vigour index

The GI value increased *i.e.* 1.47 and 1.1 fold more than the control, when 0.5 and 0.8LH were used as culture media, respectively (Table 2, Fig. 3). Whereas seedling vigour index significantly increased by 2.32 fold in 0.5LH and non-significantly increased in 0.8 LH (1.7 fold) as compared to control. Higher the GI, higher is the rate of germination (Tan *et al*., 2017). The results confirm that the seeds showed their full potential for germination in 0.5 LH in which the highest germination index and seedling vigour index was observed along with maximum pore size as observed in SEM pictures (Fig. 2B). Even the soil culture had lesser vigour index than the 0.5 and 0.8 LH. If seedbed/culture moisture is sufficient, seeds are known to complete germination (Pradeep, 2018). This proved that 0.5LH was able to provide required moisture to the seeds for their germination.

CONCLUSION

The present study introduced synthesis of lignin based biodegradable hydrogels from rice straw using PVA as the matrix template and epichlorohydrin as crosslinker. These hydrogels possessed the ability to reswell upto 4-5 cycles. Both 0.5 LH and 0.8LH proved to be efficient for *Calendula* due to their excellent germination percentage, root length, shoot length, root to shoot length ratio, germination index, seedling vigour index, fresh and dry weight values. These results can be directly applied to reduce water requirements of the plants to a great extent and it may be convenient to grow potted plants in homes, lawns and buildings.

Authors' contribution

Conceptualization and designing of the research work (RKM, GKC); Execution of lab experiments and data collection (RS); Data analysis and interpretation (RS, GKC); Preparation of manuscript (RS, RKC, GKC).

Conflict of interests

Authors declare that there is no conflict of interests.

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