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Lignin linked to slow biodegradability of urea-crosslinked starch in an anaerobic soil environment

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Abstract: For slowing fast solubility and increasing the period of urea's release in soil, urea-crosslinked starch (UcS) was prepared and applied as a slow release fertilizer. The higher environmental biodegradability of UcS is a major drawback for slow release of urea yet it provides an important challenge for large scale production and application on anaerobic farm lands. Hence, in order to reduce biodegradability of UcS, impregnation of UcS with 5–20 wt% of lignin is proposed. Lignin impregnated UcS was buried for a maximum period of 28–64 days under a constructed anaerobic soil environment. Hill's model predicted that mineralization of UcS by lignin would be reduced by 5.48%. Microbial growth on UcS was inhibited by lignin at the rate of 0.84 day^{-1} . The α -amylase activity was retarded in response to UcS impregnation with lignin at the rate of $810.94 \mu\text{g h}^{-1} \text{ g}^{-1} \text{ soil}$. Lignin also showed improvement in half-life of UcS up to 0.51 days. This work concluded that lignin impregnation was an efficient approach for improvement of UcS through increasing resistance against natural biodegraders.

Keywords: anaerobic soil; biodegradability; lignin; slow release fertilizer; urea-crosslinked starch.

1 Introduction

In 2050, the world's population is projected to be 2.3 billion people (1). This population rise is a key factor for rapid urbanization, high deforestation, increased erosion and contamination of agricultural lands. Therefore, agriculture land will not be enough for producing food in sufficient quantity in a short period of time for such fast population growth. Thus, over usage of land for crop cultivation, will require a continuous supply of nutrients in the form of fertilizer. Around 80–90% of phosphorus, 40–70% of nitrogen and 50–90% of potassium after application are lost due leaching, volatilization and run-off (2).

Urea [$\text{CO}(\text{NH}_2)_2$] is an important and a cheap source of nitrogen for plants. Urea nitrogen is absorbed by plant roots in the form of ammonium ions (NH_4^+) and nitrate (NO_3^-) (3). When urea makes contact with soil, urease enzyme in the soil acts on urea and promotes its conversion into NH_4^+ . Afterwards, NH_4^+ are used as a substrate by nitrifying bacteria and converted it into NO_3^- . Under such conditions, urea nitrogen uptake efficiency is limited to 25% up to 50% by crops (3). In anaerobic soil, denitrification action of soil microorganisms produce N_2 , N_2O and NH_3 gases from urea and these products have environmental implications. Excess of NO_3^- in water leads to water pollution (5, 6). N_2O and other oxides of nitrogen are well known as greenhouse gases, which contribute in increasing the earth's surface temperature (4). For addressing these problems conventional urea is chemically reacted with either natural or synthetic materials and are called slow release urea fertilizer. Such modifications of urea prolongs the period of release in soil, reduce the application cycles and importantly mitigate nitrogen loss (5, 6).

Low toxicity, minimal cost, biocompatibility and biodegradability are a few properties of green encapsulation materials like starch, cellulose, lignin, pectin, xanthan and alginate, which affect slow release properties of urea in soil (7–9). Starch is a water soluble plant based carbohydrate with excellent hydrogel forming properties. Starch hydrogel forms network, which have

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found important applications in agriculture like retention of soil water and as carriers for plant nutrients (10–14). Starch biodegrades easily due to attack of soil microorganisms. Microorganisms break the glycosidic bonds in starch to obtain energy and hence compromise the starch hold on slow release of nutrients. Starch with sodium alginate has been used to microencapsulate the plant growth promoting bacterium *Raoultella planticola* Rs-2 for its slow release in soil (15, 16). Release of *R. planticola* Rs-2 increases with increasing of starch concentration in the microencapsulation. Increasing of starch contents has shown an increase in biodegradability of starch and elevates the release of *R. planticola* Rs-2 from microencapsulate. Sodium alginate reduces biodegradability of starch and slows the release of *R. planticola* Rs-2 (16). The swelling, release and biodegradability of starch or alginate microencapsulated *R. planticola* Rs-2 have been impeded by adding higher bentonite contents (15).

Lignin has a filler role in starch films (17) due to its highly complex aromatic heteropolymer network of phenylpropanoid units and is composed of three phenylpropane units: guaiacyl, syringyl and *p*-hydroxyphenyl (18). Some work has been reported for developing lignin starch film and their applications in slow or controlled release fertilizer. In our previous work (5), lignin reinforcement of urea-crosslinked starch (UcS) showed reduction of 6.40–13.58% mineralization of starch with improvement in the slow release properties of urea under aerobic soil conditions. Lignin reinforced UcS can also slow the Michaelis-Menten reaction rates of soil α -amylase ~ 1.72 – 2.03 times while simultaneously inhibit the formation rates of saccharides (glucose, maltose and maltotriose) under an aerobic soil environment. The lignin reactive groups can participate in hydrogen bonding with carbonyl groups in polymers and can increase the miscibility up to 40% at lignin-polymer interface (19). Lignin presence in lignin-poly(hydroxybutyrate) (PHB) blends is known for inhibiting biodegradation in aerobic soil. Lignin could protect the biodegradation of lignin-PHB blends in soil by two approaches, (i) biochemically by protecting from attack of microorganisms on PHB, (ii) lignin molecules that coalesce on PHB surface inhibits biofilm development and cut-off microorganisms access to PHB (19). Lignin also participates in hydrogen bonding in starch films. It is reported that stilbene are water soluble lignin phenols present in kraft lignin. Stilbene reacts with starch through strong hydrogen bonding (20). Another, softwood lignin participates in adsorption of hydrolases and disrupts structural unfolding of adsorbed enzymes on lignin (21). Besides, lignin sterically hinders the attack of starch

degrading enzymes like α - and β -amylase on α -1-4 glycosidic bonds in starch and hence causes these enzymes' non-productive binding on lignin. Therefore, diffusion of starch-degrading enzymes is possibly obstructed to attack the α -1-4 glycosidic linkages within the structure of starch and it further inhibits removal of glucose or maltose from the non-reducing end of starch chains (22).

From the literature, it is established that high biodegradability of starch is a limitation for its wide application in the process of slow/controlled release fertilizer over the desired period in soil. It is expected that lignin has the ability to reduce UcS biodegradability in anaerobic soil. In case of water saturated anaerobic soil, UcS will absorb water and microorganisms, which further increase the chances of its fast biodegradability. Thus, modifying the biodegradability of UcS with lignin is considered most appropriate, which has both water repellent and microbial inhibiting effects on starch. Thus, research work is lacking, which has discussed the reduction of starch biodegradability in UcS or starch derived compositions, adopted as slow/controlled release urea fertilizer. Therefore, the objective of this research work is to reduce biodegradability of UcS through lignin impregnation of UcS under anaerobic soil microcosm.

2 Experimental

2.1 Materials

Tapioca starch (Kapal ABC, Malaysia) was purchased from the local market. Urea (46% total nitrogen) was received from Petronas Fertilizer (Kedah), Sdn., Bhd. (Malaysia). The top 20 cm of loamy sand soil was collected from a paddy field which was located at Titi Gantung (4.36°N, 100.84°E), Perak, Malaysia. The physicochemical properties of collected soil were cited in an earlier publication (23).

2.2 Preparation of lignin impregnated UcS

Tapioca starch (5 g) was heated at 80°C for 30 min in 100 ml deionized water to obtain gelatinized starch. Then, urea (2 g) and disodium tetra-borate (0.45 g) were added as a crosslinker and a catalyst, respectively. Alkaline kraft lignin (L) was added at 5, 10, 15 and 20 wt% of starch in reaction mixture. This reaction was allowed to stir for a further 3 h at 80°C to synthesize urea crosslinked starch (UcS) impregnated with lignin. The hot mixture was

poured into 12 cm × 12 cm polypropylene trays and was dried at 40°C overnight. Prepared compositions of lignin impregnated UcS were set to post cure at 120°C for 2 h. Nomenclature was assigned to lignin impregnated UcS based on lignin wt% i.e. UcS5%L, UcS10%L, UcS15%L and UcS20%L. The UcS0%L did not have lignin and it was used as a control for comparison with lignin modified UcS.

2.3 Biodegradability of lignin impregnated UcS

Soil (200 g) was saturated in pots with water to construct anaerobic conditions (width 8.5 cm × length 14 cm). A layer of water about 6–8 cm above the surface of soil was maintained to achieve anaerobic soil's redox potential ≤ 100 mV and pH ~6.4. American Society for Testing and Materials standard D6003-96 (24) was followed for the soil burial test of biodegradability for lignin impregnated UcS. Small pieces of 1.2–1.5 g of lignin impregnated UcS were placed 6–8 cm beneath anaerobic soil. Biodegradability was observed up to 60th day and tests were conducted in triplicate for various parameters as follows.

2.4 Mineralization

The CO₂ and CH₄ gases produced by lignin impregnated UcS mineralization were trapped in an air tight bottle (Figure 1). These gases were measured using a gas chromatograph (GC 2000, Shimadzu, Japan). The gas chromatograph was conditioned for column (25 m × 0.32 mm) temperature at 60°C (isothermal); flame ionization detector temperature at 200°C and injector temperature at

150°C. The 2 µl of gas mixture was injected in a split ratio 50:1 into the chromatograph column, which was carrying nitrogen carrier gas at a flow rate of 0.99 ml min⁻¹ and pressure of 30.0 kPa. External standards of CO₂ and CH₄ (mol%) were run parallel with analytical samples. The percent of mineralization was recorded by fitting CH₄ and CO₂ data into the following Equation [1] (25).

$$\% \text{Mineralization} = (\Sigma V(s) - \Sigma V(b) + V(IC) / V(\text{max})) \times 100 \quad [1]$$

Whereas, $\Sigma V(s)$ is the total volume of CO₂ and CH₄ evolved under standard conditions in mole%; $V(b)$ is the total volume of CO₂ and CH₄ evolved from blank vessel under standard conditions in mole%; $V(IC)$ is the volume of CO₂ dissolved in the test – blank soil samples under standard conditions in mole% [(CO₂ volume dissolved in test soil sample) – (CO₂ volume dissolved in blank soil sample)]; $V(\text{max})$ is the maximum theoretical volume of CO₂ + CH₄ evolved at the end of complete mineralization of lignin impregnated UcS under standard conditions. Software OriginPro version 9.0 (OriginLab Corporation, Northampton, MA, USA) was used to make calculations of kinetic estimates from mineralization data fitting into Hill's model as given in Equation [2] (26).

$$y = y_{\text{max}} \cdot \frac{t^{n'}}{k_{\text{min}}^{n'} + t^{n'}} \quad [2]$$

Here, y is the percentage of mineralization at time t (days), y_{max} is the percentage of mineralization at infinite time, k_{min} is the half-life time (days) and n' is the curve radius of the sigmoidal function. From curve fitting, r_{max} the maximum rate change of mineralization was also determined.

Figure 1: Set-up for measuring mineralization of lignin impregnated UcS.

2.5 Soil microbial biomass

The chloroform fumigation extraction method was adopted for the assessment of soil microbial biomass (27). The growth response of the soil microbial biomass was predicted through the empirical Gompertz model Equation [3].

$$X = A \exp \left\{ -\exp \left[\frac{\mu_{\max} e}{A} (\lambda - t) + 1 \right] \right\} \quad [3]$$

X is the logarithm of the relative population size of soil microbial biomass against time; A is the asymptote, representing the maximal soil microbial biomass; t is the time (days); λ is the lag phase (days); $\mu_{\max}$ is the maximum specific growth rate (day^{-1}); e is the constant.

2.6 Hydrolytic starch enzymes activities

Soil α -amylase (EC 3.2.1.1) and β -amylase (EC 3.2.1.2) enzymes' activities were assayed according to methods (28–30) and guidelines (31) published earlier. The soil was mixed with 50 ml of 20 mM phosphate buffer (pH 6.9), which was composed of 6.70 mM NaCl (activator of α -amylase enzyme). In the case of β -amylase enzyme, the soil was mixed with 50 ml of 16 mM acetate buffer (pH 4.8). The assay was initiated with 2 ml of soil extract, 0.5 ml of 2% potato starch solution, 3.5 ml of phosphate buffer in a test tube. Samples were incubated for 2 h at 30°C. A control reaction of the enzyme assay was also set up parallel in which soil was added at the end of assay's incubation period.

Release of the reducing sugar was immediately measured on a UV-visible spectrophotometer (Shimadzu, Japan) at wavelength of 534 nm. For the activity assay of α -amylase and β -amylase, glucose and maltose standards, respectively, were run at 0, 18, 36, 54, 72, 90, 108 and 126 μM . Activity was monitored using following Equation [4] as given below.

$$\text{Activity } (\mu\text{g h}^{-1}\text{g}^{-1}\text{ dw soil}) = \frac{\text{Concentration } (\mu\text{g}) \times \text{reaction volume (ml)}}{\text{Homogenate volume (ml)} \times \text{Time (h)} \times \text{soil dw (g)}} \quad [4]$$

Reaction rates of enzymes were determined by the Michaelis-Menten equation [5].

$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad [5]$$

$[S]$ is the substrate concentration (μg), $V_{\max}$ is the reaction rate of enzyme, K_m is the substrate affinity constant.

2.7 Starch biodegradability in lignin impregnated UcS

Starch biodegradability in lignin impregnated UcS was also analyzed using the perchloric extraction technique (32). One gram of freeze dried soil was washed with 5 ml of 80% hot ethanol for 30 s to remove the soluble sugars. After this, 4 ml of distilled water and 5 ml of 52% cold perchloric acid were added to the soil sample prior to extraction in an ice cooled water bath for 1 h. In this extract, the absorbance of starch was monitored at 625 nm on a UV-visible spectrophotometer (Shimadzu, Japan). A calibration curve was established with a known concentration of starch standards in the range of 0–200 $\mu\text{g l}^{-1}$. The unknown starch concentration was determined in soil samples by plotting its value on the calibration curve. The first-order reaction rate constant of starch decay was predicted through Equation [6].

$$C_{s,t} = C_{s,0} \cdot e^{-k_s t} \quad [6]$$

$C_{s,0}$ (μg) is the initial concentration of starch; $C_{s,t}$ (μg) is the concentration of starch at a particular time t ; k_s (day^{-1}) is the kinetic constant of starch decay. The half-life ($t_{1/2}$) was predicted through Equation [7].

$$t_{1/2} = \frac{\ln 2}{k_s} \approx \frac{0.693}{k_s} \quad [7]$$

The products of starch biodegradation like glucose, maltose and maltotriose were derivatized with 1-phenyl-3-methyl-5-pyrazolone to know the yield, reaction rate and starch bio-fragmentation (BF). These derivatized products were separated and quantified through reverse phase high pressure liquid chromatography (Agilent, 1100) which was equipped with a Zorbax Eclipse XDB-C18 RP-HPLC column (250 mm length, 4.6-mm internal diameter and 5- μm particle size, Agilent, USA) at 30°C. The products were eluted in an isocratic mode with a mobile phase containing 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v%; pH 7.1) with a flow rate of 1 ml min^{-1} . A UV detector was set at a wavelength of 245 nm.

The BF of starch was calculated for each composition of lignin impregnated UcS by following Equation [8] (33).

$$\% \text{ BF} = \frac{\text{Produced glucose polymer DP}}{\text{Theoretical glucose equivalent}} \times 100 \quad [8]$$

The total % BF is presented by Equation [9] as below.

$$\text{Total \% BF} = \sum_{\text{DPn}} (\text{BF}) \quad [9]$$

Theoretically, the amount of total glucose equivalent in each lignin impregnated UcS was determined by using the phenol-sulfuric acid method (34).

2.8 Statistical analysis

Mean data was tested for significant difference at $p < 0.05$. One-way analysis of variance (ANOVA) was applied to test the significant difference of lignin in UcS on the reaction rates of soil enzymes which degrades starch. A one-way repeated measures ANOVA followed by a *post hoc* Dunnett's test was applied to predict the significant effect of lignin impregnation of UcS on the mineralization, microbial biomass, starch biodegradability and release of saccharides. All statistical tests were run on graphical and analysis software (OriginPro version 9.0, OriginLab Corporation, Northampton, MA, USA). The error mean square (MSE) was also estimated by the following Equation [10].

$$\text{MSE} = \frac{\text{Sum (experimental values - predicted values)}^2}{\text{degree of freedom}} = \frac{\sum (Y_i - \hat{Y}_i)^2}{n - 1} \quad [10]$$

3 Results and discussion

3.1 Mineralization kinetics

Figure 2 shows Hill's model fitting to mineralization data of UcS with different percent loading of lignin. The estimates of mineralization determined through Hill's model are shown in Table 1. The predicted and experimental

Figure 2: Hill's model fitting to mineralization data of lignin impregnated UcS.

response of mineralization were in good agreement ($R^2 > 0.967$ and $\text{MSE} < 4.36\%$). The $y_{\max}$ values of lignin impregnated UcS were decreased from a maximum of 32.98% to a minimum of 27.50% (difference of 5.48%) in response to increasing lignin contents. The $k_{\min}$ showed the same trend as $y_{\max}$ and found a reduction of a maximum of 18.85 days to a minimum of 17.56 days (difference of 1.29 days). However, $k_{\min}$ found for UcS with 10% lignin was 19.10 days, which showed an increase of $k_{\min}$ 1.01 times compared to the control. The $r_{\max}$ for 5–15% lignin impregnated UcS was observed a maximum of ~ 0.66 times to a minimum of 0.62 times (difference of 0.40 times) higher than the control. The $r_{\max}$ of 20% lignin impregnated UcS reduced ~ 0.93 times, which was more than other compositions. In addition, higher lignin percentage can cause two thermodynamically immiscible phases in UcS, which are represented as hydrophobic (lignin) and hydrophilic (starch) (17). This phase separation increases the plasticizing effect of water in soil through the interaction with the exposed portion of starch at the interface of two phases in lignin impregnated UcS. That way, water increases the void volume and accessibility of soluble starch to soil microorganisms at a higher lignin impregnation ratio in UcS. Thus, the structural heterogeneity of lignin influenced the noticeable variation in $k_{\min}$ and $r_{\max}$ of 10% and 20% lignin modified UcS.

3.2 Soil microbial biomass

Figure 3 shows that the soil microbial biomass required 6 days to reach maximum growth and after that decline until 28 days for all biodegraded lignin impregnated UcS. Growth of microbial biomass was inhibited initially for a short period up to 4 days by lignin loadings. Soil microbial biomass was observed 1.05 times to 1.24 times higher for lignin impregnated UcS as compared to control UcS over the period of biodegradation in anaerobic soil. A significant difference of lignin on growth of soil microbial biomass was predicted by one-way repeated measures ANOVA ($F_{1,8} = 20.41$, $p < 0.001$).

Higher soil microbial biomass in the presence of lignin on 6th day and the onward decline could be explained based on the macromolecular structure, loading amount and biodegradation rates of lignin and starch. Starch is amorphous and its glycosidic bonds break faster than the oxidative cleavage of lignin delocalized bonds. Hence, initial soil microbial biomass growth up to 4 days is driven by starch biodegradation. While after 4 days of biodegradation, an excess of resistant lignin accumulates along with a minimal amount of resistant starch. This causes the decline

Table 1: Hill's model derived estimates of mineralized lignin impregnated UcS in anaerobic soil.

Films	Time (days)	$y_{\max}$ (%)	$r_{\max}$ (% day ⁻¹)	$k_{\min}$ (days)	n'	MSE	R^2
UcS0%L	21	32.98	2.08	18.89	7.28	0.73	0.996
UcS5%L	21	27.45	1.39	18.85	5.31	0.14	0.998
UcS10%L	21	26.39	1.30	19.10	4.59	0.42	0.997
UcS15%L	21	26.30	1.39	18.50	5.49	0.14	0.998
UcS20%L	21	27.50	1.94	17.56	7.15	4.36	0.967

Figure 3: Soil microbial biomass grown on lignin impregnated UcS under anaerobic soils.

in growth of soil microbial biomass. This could be explained as after starch utilization, soil microbial biomass consumes a small fraction of lignin until they meet growth inhibitory fraction of lignin in lignin reinforced UcS. This view is supported with the presence of autochthonous species in soil, which are well known for the high efficiency of conversion of high energy organic macromolecules (i.e. lignin)

into soil microbial biomass (35). Such transient microbial activity, then ends up with more resistant residues mainly the lignin, which further slow down the microbial growth (Figure 3, 6th–28th day), due to a few classes of bacteria and fungi involved in lignin's biodegradation (35). This process is initiated by starch. When starch is oxidized under reducing soil conditions, lignin acts as a terminal electron acceptor for starch. This observation is in agreement with earlier work (36), where xylose (a carbohydrate) increased the growth of lignin-degrader soil bacterium, *Enterobacter lignolyticus* SCF1 by utilizing lignin as a terminal electron acceptor under controlled laboratory anaerobic media conditions. In addition, low oxidation state of starch is due to its low standard enthalpy of combustion $\sim 4.15 \text{ kcal g}^{-1}$ (37) in comparison with the higher enthalpy of combustion of lignin $\sim 6.37 \text{ kcal g}^{-1}$ (38). Higher enthalpy of combustion turns lignin carbon into soil microbial biomass more slowly as compared to starch in biodegraded UcS. Although, growth of the soil microbial biomass was higher with the increase of lignin contents but a decline was obvious after 6th day of biodegradation.

Gompertz's model fitting to the experimental data ($R^2 = 0.994$) is shown in Figure 4 to predict growth kinetics

Figure 4: Gompertz's model fitting to soil microbial biomass grown on lignin impregnated UcS.

Table 2: Gompertz's model estimates for soil's microbial biomass determined for lignin impregnated UcS.

Compositions	A	$\mu_{\max}$ (day ⁻¹)	λ (day)	t_i (day)
UcS0%L	2.391	1.814	0.220	0.705
UcS5%L	2.430	1.367	0.120	0.774
UcS10%L	2.507	1.237	0.168	0.914
UcS15%L	2.571	1.056	0.150	1.045
UcS20%L	2.660	0.978	0.138	1.139

of soil's microbial biomass for lignin impregnated UcS as shown in Table 2. The $\mu_{\max}$ (day⁻¹) and λ (days) were found to be negatively correlated with the amount of lignin added into UcS. However, $\mu_{\max}$ and λ both were negatively correlated with estimated values of A and t_i . The lower $\mu_{\max}$ and corresponding high values of A showed a fast turnover of carbon substrate into the soil's microbial biomass due to lignin's electron acceptor properties (36) and high enthalpy of combustion compared to starch. These two properties of lignin could be responsible for the high prediction of soil's microbial biomass growth on lignin impregnated UcS as compared to control UcS.

3.3 Hydrolytic enzymes activity

Figure 5 shows that lignin inhibits the Michaelis-Menten reaction rates of soil α -amylase after lignin impregnation of UcS. Table 3 shows Michaelis-Menten reaction rate constants of soil α -amylase. The $V_{\max}$ of soil α -amylase declined with the increase of lignin percentages. The $V_{\max}$ of soil α -amylase was inhibited 1.23 times to 1.44 times by lignin impregnation of UcS as compared to control UcS. The K_m of soil α -amylase decreased 1.11 times to 1.31 times in

Figure 5: Effect of lignin impregnation of UcS on Michaelis-Menten reaction rates of soil α -amylase.

response to lignin, which was correlated with an increase in affinity of the soil α -amylase for lignin impregnated UcS. The k_{cat} of soil α -amylase was minimized by increasing lignin contents in UcS. The k_{cat} of the soil α -amylase was ~1.28 times to 1.34 times less for lignin impregnated UcS in comparison with the soil that was amended with control UcS. The K_m and $V_{\max}$ of soil α -amylase showed an inhibitory effect of lignin on the activity of soil α -amylase was a mixed linear type. Thus, the mixed linear type of inhibition implicates that lignin favorably binds the soil α -amylase-starch (substrate) complex instead of binding directly to soil α -amylase in lignin impregnated UcS. Similar, a mechanism has been reported for cellulose-based inhibition of soil α -amylase activity (39).

Lignin stalled the $V_{\max}$, K_m and k_{cat} of soil α -amylase. This clearly showed that starch was less bioaccessible and more protected in lignin impregnated UcS. This is in agreement with reported findings in literature that 0–0.48 μM of lignin can bind with α -amylase (30). Lignin binding with α -amylase could disrupt hydrogen bonds in the internal helical structure of α -amylase. In current research work, coupling of α -amylase and lignin in lignin impregnated UcS can inhibit starch depolymerization. Such adsorption and disruption action of lignin on enzyme was confirmed by finding an increase in soil α -amylase affinity (lower K_m). This effect likely limited the diffusion of soil α -enzyme to access starch within lignin impregnated UcS. This was in accordance with other findings in the literature that substrates below a certain threshold could restrict the substrate availability for biodegradation due to mass-transfer limitations, which have been reported under porous medium (like soil) (40).

3.4 Starch biodegradability

Figure 6 shows the starch biodegradability in lignin impregnated UcS. Starch was biodegraded in an exponential fashion and lost a maximum until 6 days in lignin impregnated UcS. Up to 6 days, 5–15% of lignin impregnation of UcS effectively reduced starch biodegradability ~0.95 times to 0.98 times more as compared to control UcS. However, UcS20%L lost more starch. The reason for this deviation is possibly related to the poor mechanical resistance at higher lignin percent (17). This could be due to water mediated starch erosion, which supersedes the amylase driven biodegradability under oxygen limited conditions. Lignin-impregnated UcS showed no significant reduction in starch biodegradability (one-way repeated measures ANOVA, $F_{1,9}=2.38$, $p>0.05$). In Table 4, the reduction of k_s and increase of $t_{1/2}$ of starch

Table 3: Michaelis-Menten reaction rate constants of soil α -amylase for different lignin impregnated UcS.

Films	$V_{\max}$ ($\mu\text{g h}^{-1} \text{g}^{-1} \text{ soil}$) Mean $\pm$ SE	K_m (μg) Mean $\pm$ SE	K_{cat} ($\text{h}^{-1} \text{g}^{-1} \text{ soil}$)	R^2	Dunnett's test p-Values
UcS0%L	1834.58 $\pm$ 38.84	9616.03 $\pm$ 2205.30	0.190	0.987	–
UcS5%L	1271.44 $\pm$ 69.98	8587.31 $\pm$ 1472.08	0.148	0.991	0.768
UcS10%L	1160.63 $\pm$ 66.74	8006.48 $\pm$ 1463.67	0.144	0.989	0.636
UcS15%L	1097.03 $\pm$ 63.07	7795.90 $\pm$ 1435.83	0.140	0.988	0.539
UcS20%L	1032.64 $\pm$ 60.10	7312.58 $\pm$ 1388.02	0.141	0.987	0.473
Blank soil	386.38 $\pm$ 21.76	4812.65 $\pm$ 981.43	0.080	0.956	0.031 ^a

^aSignificant difference ($p < 0.05$).

Figure 6: Effect of lignin impregnation on starch biodegradability in UcS.

Table 4: Effect of lignin on k_s and $t_{1/2}$ of starch in lignin impregnated UcS.

Films	First-order Eq. $C_{s,0} e^{-k_s t}$	MSE	R^2	$t_{1/2}$ (days)
UcS0%L	$19.53e^{-0.907t}$	1.048	0.996	0.764
UcS5%L	$24.84e^{-0.773t}$	0.917	0.998	0.896
UcS10%L	$16.79e^{-0.746t}$	0.979	0.997	0.929
UcS15%L	$17.50e^{-0.728t}$	0.921	0.997	0.952
UcS20%L	$19.40e^{-0.543t}$	0.898	0.998	1.276

were noticed after addition of lignin into UcS. The $t_{1/2}$ of starch was improved ~ 1.17 times– 1.66 times.

3.5 Release of Saccharides

The maximum yield of glucose (Figure 7A), maltose (Figure 7B) and maltotriose (Figure 7C) was exponentially increased only up to 4 days in all lignin impregnated UcS and control UcS. After this period, an exponential decline was also noticed for saccharides up to 28 days. The yield of each saccharide was correlated negatively with lignin

Figure 7: Effect of lignin on yields of different saccharides: (A) glucose, (B) maltose, (C) maltotriose in lignin impregnated UcS.

percent increase in UcS. One-way repeated measures ANOVA showed a highly significant yields of glucose ($F_{1,8} = 24.33$, $p < 0.001$), maltose ($F_{1,8} = 12.53$, $p < 0.001$) and

Table 5: Reaction kinetic rates for saccharides formation and depletion in biodegraded lignin impregnated UcS.

Saccharides	Films	Saccharides formation rates, k_f (day ⁻¹) (1–4 days)		Saccharides depletion rates, k_d (day ⁻¹) (4–28 days)	
		First-order kinetic rate equation	R^2	First-order kinetic rate equation	R^2
Glucose	UcS0%L	$y = 872.91 - 868.86e^{-0.881t}$	0.998	$y = 146.10 + 1099.97e^{-0.108t}$	0.999
	UcS5%L	$y = 789.62 - 788.53e^{-0.957t}$	0.999	$y = 111.93 + 986.90e^{-0.098t}$	0.999
	UcS10%L	$y = 671.089 - 674.45e^{-0.988t}$	0.997	$y = 59.14 + 863.96e^{-0.089t}$	0.994
	UcS15%L	$y = 646.99 - 643.46e^{-0.845t}$	0.999	$y = 43.28 + 819.65e^{-0.088t}$	0.998
	UcS20%L	$y = 640.17 - 638.40e^{-0.426t}$	0.999	$y = 49.12 + 674.70e^{-0.082t}$	0.998
Maltose	UcS0%L	$y = 1360.96 - 1356.94e^{-1.291t}$	0.997	$y = 210.15 + 4231.92e^{-0.321t}$	0.999
	UcS5%L	$y = 1264.85 - 1263.71e^{-1.250t}$	0.999	$y = 161.88 + 3603.23e^{-0.295t}$	0.998
	UcS10%L	$y = 1217.56 - 1217.22e^{-1.165t}$	0.999	$y = 136.47 + 3277.15e^{-0.283t}$	0.995
	UcS15%L	$y = 1186.25 - 1187.14e^{-1.021t}$	0.999	$y = 149.05 + 3501.97e^{-0.313t}$	0.996
	UcS20%L	$y = 1043.56 - 1045.93e^{-1.089t}$	0.999	$y = 132.303 + 2455.05e^{-0.261t}$	0.994
Maltotriose	UcS0%L	$y = 1598.96 - 1607.84e^{-0.490t}$	0.998	$y = 284.40 + 3977.79e^{-0.325t}$	0.999
	UcS5%L	$y = 1847.73 - 1858.17e^{-0.327t}$	0.998	$y = 193.89 + 4111.48e^{-0.319t}$	0.995
	UcS10%L	$y = 2121.50 - 2129.69e^{-0.231t}$	0.999	$y = 137.70 + 3827.17e^{-0.304t}$	0.999
	UcS15%L	$y = 4227.93 - 4237.35e^{-0.088t}$	0.999	$y = 82.47 + 3803.30e^{-0.297t}$	0.999
	UcS20%L	$y = 2969.83 - 2976.26e^{-0.117t}$	0.999	$y = 86.05 + 3189.01e^{-0.286t}$	0.999

maltotriose ($F_{1,8} = 11.29$, $p < 0.001$). A *post hoc* Dunnett's test of pairwise means' comparison showed a highly significant difference ($p < 0.001$) in yields of released saccharides. The maximum yield of saccharides followed from highest to lowest as maltotriose > maltose > glucose. This finding has confirmed that large fragments were produced faster during biodegradation events in all compositions of lignin impregnated UcS. This observation can be explained as more large fragments of saccharides were produced because lignin protected the attack on the glycosidic bond in starch of UcS. This is an indication of the slow biodegradability of starch in UcS when lignin was added. In the absence of lignin as in control UcS, more glycosidic bonds are attacked and hence a small number of large fragments of saccharides are produced.

Table 5 shows that first-order reaction rates of saccharides' formation (1–4 days) were decreased for glucose ~1.08–0.48 times; maltose ~0.96–0.84 times; maltotriose 0.66–0.23 times as compared to control UcS. Formation rates for saccharides were recorded as the lowest to the highest in the following order: maltotriose > glucose > maltose. Therefore, first-order reaction rates of saccharides explained starch disintegration mainly catalyzed by soil α -amylase. Based on these findings, it is anticipated that slow formation rates of long chain saccharides (maltotriose and above) could be due to fast conversion of starch into short chain saccharides.

Table 5 also shows first-order reaction rates for saccharides' depletion (4–28 days). Depletion of released saccharides was slowed more after lignin's loading into UcS. Lignin slowed the depletion of glucose ~9.26%–24.07%;

maltose ~8.10%–18.69%; maltotriose ~1.85%–12.00%. The fast depletion of maltotriose in contrast to glucose and maltose could be an indication of the lower amount of time required for conversion of large fragments of starch into small fragments. This was related to the breakdown of a greater number of glycosidic linkages in long fragments of starch. In Figure 8, a decline was observed in total percentage of biofragmentation after increasing the lignin amount in UcS. The biofragmentation was 83.50%–94.83%, which was found to be less than control UcS with no lignin.

Hydroxyl groups present in lignin's structure take part in hydrogen bonding with hydroxyl group of starch in UcS (22). Stilbenes are small fractions of soluble hydrophilic phenolic compounds which exists in lignin fractions.

Figure 8: Effect of lignin on starch biofragmentation in lignin impregnated UcS.

Stilbenes are reactive in nature and responsible for lignin reactivity with starch through developing hydrogen bondings (20). In a recent study on the biodegradability of lignin reinforced UcS in aerobic soil, lignin obstructs the diffusion of starch-degrading enzymes into UcS and hence prevents the enzyme's attack on α -1-4 glycoside bonds (22). In the current study, analysis supports the effect of lignin towards disengaging starch and soil α -amylase coupling. In this way it is possible that lignin can protect the starch biodegradation in UcS and hence, slow the rate of biofragmentation as reported in Table 5 and Figure 8.

4 Conclusion

Urea entrapped in UcS is known for slow release of the urea in soil. But starch present in UcS biodegrades in soil very fast and causes an earlier release of urea than expected. Hence, this work addresses this important issue and demonstrates in detail the minimization of starch biodegradation in UcS under anaerobic soil. Lignin proved a good inhibitor for slowing biodegradability of UcS to solve this problem. The lignin being heterogeneous and hydrophobic in nature contributed to the declining mineralization of UcS up to 5.48%. Lignin effectively inhibits the reaction rate of soil α -amylase as K_{cat} reduces from 1.28 times up to 1.34 times in anaerobic soil added with lignin impregnated UcS than soil amended with UcS. Lignin inhibition of soil α -amylase further reveals lignin favorably binds the soil α -amylase-starch (substrate) complex instead of binding directly to soil α -amylase in anaerobic soil. The lignin sustained more growth of the soil's microbial biomass up to 6 days after some initial growth on starch up to 4 days in lignin impregnated UcS. Higher enthalpy of combustion of lignin as compared to starch in UcS is possibly responsible for slow conversion of lignin's carbon into soil's microbial biomass as compared to starch in biodegraded lignin impregnated UcS. Starch in lignin impregnated UcS exponentially biodegrades in maximum 6 days due to soil-water mediated starch erosion, which supersedes the soil amylase driven biodegradability under oxygen constrained environment. The first-order of reaction implies the slow formation of glucose, maltose and maltotriose in lignin impregnated UcS with an increase of lignin amount. The biofragmentation of starch in lignin impregnated UcS was noticed to be 83.50%–94.83%, which was less than found in control UcS. Lignin's effect on reducing the release of saccharides and hence protecting starch biofragmentation is the outcome of lignin's contribution in disengaging the starch and soil α -amylase coupling. This could lead to concluding that lignin can protect the starch biodegradation in

UcS and further improve usability of UcS as a slow release urea formulation under an anaerobic soil.

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