Effect of charring on rice grain morphology and carbon isotopic composition

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Rice cultivation over Asia has several thousand years of history. Adequate water availability is a prime factor for the cultivation of rice in this region. The remains of rice at the archaeological sites, therefore, provide an indirect clue on rainfall in this region. The stable isotopic compositions in remains of rice grains allow estimation of rainfall condition during rice cultivation. Often, such remains found at the archaeological sites suffer from the process of charring, which is likely to modify the original isotopic signature. Here, we performed charring experiments on rice grains at two different temperatures, i.e. 230°C and 250°C and documented the changes in the morphology and carbon isotopic composition (δ^{13}C). A noticeable morphological shift was registered in the samples with progressive duration and temperature of charring. Further, cellulose was extracted and analysed for δ^{13}C. Our results showed that the shift in δ^{13}C observed for charred rice was relatively lower as compared to that observed in other cereals.

Keywords: Charring, palaeoclimate proxy, rice, stable carbon isotope.

CHARRED cereals and pulses are commonly found as remains in archaeological sites and can serve as important organic remains for deducing crop growing conditions, including agricultural practices adopted by the population at different settlement sites of the past civilizations[1–5]. The process of charring involves partial oxidation due to incomplete combustion of original organic remains of food grains at archaeological sites. The degree of preservation of food grains is always a major concern while using them as materials to trace the original climate conditions during deposition. The carbon isotopic composition (δ^{13}C) of plant tissues, including the remains of seed grains, provides information about the stomatal conductance and mechanism of carbon fixation in a plant, which in turn reflects the availability of water in the environment[6,7]. Availability of water is linked with factors such as precipitation and relative humidity, which control the opening/closing of stomata to regulate the loss of water, thereby governing the carbon isotopic discrimination in the plant during synthesis of organic matter[7]. Using isotopes as tracers, past climatic shifts have been traced in numerous studies by measuring δ^{13}C and oxygen isotopic composition (δ^{18}O) of plant organic remains[8–10]. For example, rice grains from archaeological sites of Harappan civilization[10] and tree rings of Aleppo pine (*P. halepensis*) from the archaeological sites of East Iberian Peninsula[8] allowed reconstruction of the hydroclimatic condition, besides deduction of agricultural practices[8,10]. The story of rice domestication over Asia dates back to 10,000 years when human settlements of Yangtze river valley and of the Ganges and Indus river valleys initiated cultivation of rice for food[11–14]. Rice cultivation was widely practiced in the settlements of the Harappan civilization that existed between 3200 and 1000 year BC in northern Indo-Gangetic plains of South Asia[15]. This is evident by the presence of well preserved charred rice grains in all the three phases of the Harappan civilization[13], with the oldest evidence of rice gathering and cultivation practices documented at the neolithic site of Lahuradewa, India[14]. Despite the widespread availability of rice in archaeological sites over Asia, it has seldom been used to obtain a quantitative estimate of the climatic conditions[10].

Application of stable isotope technique to reconstruct climate using archaeobotanical remains necessitates preservation of the original isotopic composition. Preservation of archaeobotanical remains can take place by charring, as mentioned earlier. The process of charring involves subjecting the organic matter to heat in a reducing environment for a prolonged period. This causes removal of volatiles from the bulk, while the starch and protein undergo a complex structural transformation leading to darkening of grain colour by Maillard reaction[16–18]. Chemically, the rice husk is made up of cellulose, lignin and silica[19] and grain kernel is composed of starch, lipid and protein. The Maillard reaction takes place between sugar and amino acids, leading to the formation of compounds with higher molecular weight or complex heterocyclic structures. Chemical breakdown of those charred grains is difficult due to the inertness of the complex organic structure, making those grains resistant to the

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process of biological degradation; allowing preservation of original composition[20,21]. A number of earlier studies addressed the effect of charring on other botanical taxa upon prolonged heating at higher temperatures; mimicking burial conditions[17,22,23]. These studies investigated morphological transformation in the cereal grains and their isotopic composition. Summary of observations suggests that in some instances the carbon isotopic composition of the bulk charred cereal grains and wood deviate from the original composition upon charring, which is a result of the retention of ^{13}C depleted compounds such as lignin in the organic matter as compared to the relatively ^{13}C enriched polysaccharides that might be lost during thermal degradation[24,25]. This provides the background on why the effect of charring and the extent of preservation of biochemical components needs to be evaluated for rice grains. A previous study has shown the utility of carbon isotopic composition of rice grain to record climate conditions[6]. A subsequent study argued in favour of preservation of original isotopic composition in charred rice grains and further used this proxy record to deduce past hydrological conditions that were found to be consistent with records in other climate proxies[10].

In the present study, we conducted controlled charring experiments on rice grains and compared our observations with other cereals. The aim of the present study is to understand the effect of charring process on rice grain morphology and carbon isotopic composition of the husk, caryopsis and preserved cellulose. For this, rice grains of variety IR64 sampled from Tumkur district of Karnataka, India were selected. The samples comprising several grains were subjected to two different temperature conditions: 230°C and 250°C in a poorly ventilated setup created in the laboratory, mimicking the field condition.

Materials and method

Charring

The sample grains were processed for any presence of dust and oven dried prior to charring. Then sub-samples comprising of 15–20 grains were taken and weighed prior to the charring experiment. Charring was done under two temperature conditions; 230°C and 250°C; for different durations, i.e. 2, 4, 8, 16 and 24 h at 230°C. At 250°C, samples were charred for 2, 4 and 6 h.

The sample preparation procedure involved enclosing sample replicates in aluminum foil and placing them inside a borosilicate glass crucible filled with sand[22]. Then the crucible was further wrapped with aluminum foil to minimize the supply of air. The crucibles were placed inside a muffle furnace, where the first set of target materials was subjected to a temperature of 230°C. The temperature was gradually increased inside the muffle furnace at the rate of 1.5–2°C/min until it attained 230°C and then kept for 2, 4, 8, 16 and 24 h. The second set of experiments was performed likewise where the temperature was increased up to 250°C, keeping every other parameter constant. After completion of the experiments, the final weight of the samples in individual cases were measured. Photomicrographs were also taken under stereo-microscope fitted with a camera. These charred samples were further processed for isotopic analysis and cellulose extraction.

Cellulose extraction

Uncharred samples: Cellulose extraction from the husk of rice grains was done following the well-accepted technique that is commonly used for wood sample[26]. The procedure involves removal of about 500 mg of husk from assorted rice grains followed by washing and drying. The husk was then powdered and loaded in a Borosil thimble placed inside a clean beaker. A solution containing 1.4% (w/v) sodium chlorite and 0.97% (v/v) acetic acid was poured into the beaker until the thimble was submerged completely. The beaker was covered with aluminum foil to avoid any contamination. The entire setup was placed in an ultrasonic bath at 70°C for a period of 5 h, inside a fume hood. The solution was refreshed at intervals of 1 h. The thimble was retrieved at the end of 5 h and the yellowish-white residue on the glass fret was washed with ample amount of hot deionized water followed by cold deionized water. The washing procedure was repeated until the bulk sample turned white. The thimble was then placed in a clean beaker and 10% (w/v) sodium hydroxide was added. The setup was put in the ultrasonic bath for another 1 h at 80°C, followed by washing of the extracted residue with cold deionized water. After this step, the residue was treated with 17% (w/v) sodium hydroxide in a similar manner and placed in the ultrasonic bath for 1 h in 80°C. After retrieving the thimble, the residue was washed with 17% (w/v) sodium hydroxide, then treated with cold deionized water and finally with 1% (w/v) hydrochloric acid till the solution achieved neutral pH. The final product, i.e. cellulose was then oven dried.

Charred samples: Cellulose present in the charred grains is susceptible to degradation on prolonged exposure to acetic acid. Therefore, the protocol of cellulose extraction was modified in the case of the charred samples in terms of duration of the different chemical treatments. Charred grains were visually examined for any visible morphological transformation and the best preserved grains were identified for cellulose extraction. Grains charred at 230°C for 16 h were the best analogue of the archaeological rice grains recovered from several sites across Asia. This was ascertained based on visual inspection and microscopic similarity of grain morphology. Therefore, charred samples generated at 230°C for 16 h were further processed for cellulose extraction. Outside hull/husk of the charred grains was removed,

powdered and about 350 mg was weighed into a thimble. The thimble was then placed in a clean beaker containing a solution of 0.0012% sodium chlorite and 1.8% acetic acid. Standardization of methodology was done by trial and error method. To avoid contamination the beaker was covered with aluminum foil that was perforated to let go of the acid/chlorine gas and the entire set up was heated in an ultrasonic bath at 70°C for 3.2 h, inside the fume hood. A fresh solution was added multiple times at an interval of 1 h until completion of extraction treatment covering 3.2 h. Subsequently, the thimble containing powder was removed from the beaker and washed with hot deionized water followed by cold deionized water. If the colour turned whitish or yellowish white, we proceeded to the next step, otherwise the procedure was repeated until the desired colour residue was obtained. The thimble was retrieved and placed in a fresh beaker with 10% sodium hydroxide (w/v), where the colour of residue turned brown. The whole set-up was kept in an ultrasonic bath for 25 min at 80°C. The residue was then washed using ample amount of cold deionized water followed by treatment with 17% sodium hydroxide (w/v). The whole set up was then kept in the ultrasonic bath for 10 min at room temperature. Once the husk powder turned light brown, the liquid was drained off and the residue was washed using cold deionized water. After this step, the sample was initially washed using 17% sodium hydroxide (w/v), followed by a generous rinsing with cold deionized water and 1% hydrochloric acid (w/v) and finally with ample amount of cold deionized water until the pH turned neutral. The residue, i.e. cellulose was kept overnight for drying inside an oven at a temperature of 75–80°C.

Characterization of cellulose

XRD analysis: X-ray powder diffraction (XRD) technique was used for identification of the cellulose extracted from both the uncharred and charred rice grain husk samples. This technique provides information about the unit cell dimensions of the crystal structure. XRD analysis was performed on the cellulose powders at the central facility of the Indian Institute of Science, Bangalore. The sample powders were placed in a sample holder and levelled to provide a planar surface for uniform X-ray exposure. The sample analysis was performed using X-ray diffractometer (D8-Advanced Bruker AXS GmbH) at room temperature (RT) using a monochromatic CuKα radiation source in the step-scan mode 2θ angle ranging from 10° to 70° with a step of 0.04° at 5 min of time interval.

FTIR analysis: The analytical technique of FTIR (Fourier Transform Infrared) spectroscopy is used to identify organic, polymeric and inorganic materials. In this method, the infrared light is used to scan test samples and observe their chemical properties. We used the Perkin-Elmer FTIR spectrometer Fourier transform infrared spectra to identify cellulose in both charred and un-charred husk samples. Sample powder was added with potassium bromide (KBr) to generate a pellet using a compressive device. Then the pellet was used for analysis. We also analysed a commercially available pure cellulose powder to compare the spectra generated by charred and uncharred cellulose from our experiments.

Analysis of stable isotopic composition: The husk and caryopsis were isolated and crushed homogeneously into fine powder using agate mortar and pestle (125–250 μm in size). Samples of cellulose (both from uncharred and experimentally generated charred rice husk samples at 230°C for 16 h, husk and caryopsis were analysed for carbon isotopic composition. Sample powders were packed into tin capsules within the weighing range of 150–170 μg and analysed using FLASH 2000 Elemental Analyser (Thermo Scientific). The combustion reactor of FLASH 2000 comprised chromium (III) oxide combustion catalyst and silver cobaltous oxide at 1050°C and the reduction reactor comprised of reduced copper grains at 680°C. Samples were analysed in duplicates and the isotope ratio values were expressed using the delta notation (eq. (1)) in per mil (‰), relative to the standard Vienna Pee Dee Belemnite

$$\delta^{13}\mathrm{C}\ (‰) = \left(\frac{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{Sample}}}{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{Standard}}} - 1 \right) \times 1000. \quad (1)$$

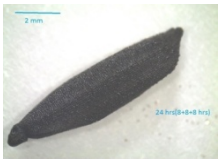
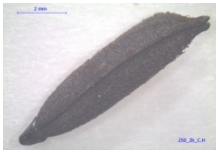
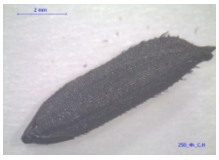
Two internal working reference materials which includes a rice grain powder (referred to as Oasis_rice 1) and a glucose powder with $\delta^{13}C_{VPDB}$ values of –27.67‰ and –10.45‰ respectively[6], were analysed along with the samples for assigning δ^{13}C values to the samples. These working reference materials were assigned values based on repeated measurements with international reference material of IAEA CH-6. The analytical precision was 0.05‰ based on repeat analyses of the glucose reference material.

Results and discussion

Effect of charring on morphology

In each of the charring experiments that involved temperature of 230°C and 250°C, we observed a progressive change in colour of the grains from white to brown to black with the duration of charring. The samples turned black in a relatively shorter time span for the experiment conducted at higher temperature as compared to the lower temperature. The change in colour is due to Maillard reaction as explained earlier where complex organic

Table 1. Display of photomicrographs of rice grains subjected to different charring conditions

Duration (h)	Micro-photograph	Description
230°C		
2		Perfect, colour is as of uncharred grain.
4		Epidermis is intact, rachilla is present, epidermal hairs are partially preserved, brown in colour.
8		Epidermis is preserved, smoother surface, rachilla is partially preserved, dark brown in colour.
16		Epidermis is fragile, rachilla is partially preserved and sterile lemmae degenerate, hairs almost removed, darker and smoother surface and black in colour.
24		Epidermis becomes very brittle, rachilla is absent (if preserved very fragile), hairs completely removed, fragile grain, black in colour.
250°C		
2		Epidermis is intact, dark brown in colour, rachilla and sterile lemmae are preserved, hairs partially preserved.
4		Epidermis is intact, darker in colour, rachilla is partially preserved, hairs partially preserved.
6		Epidermis is fragile, rachilla is partially preserved and sterile lemmae are absent, hairs mostly absent, dark black in colour.

molecules are generated from reaction between starch and protein and are resistant to any further degradation in case of archaeobotanical samples[16].

The surface of grain husk is characterized by hair-like appendages. These appendages were found intact in samples that were charred at 230°C up to 8 h, but absent in samples that were subjected to the same charring temperature for a longer duration of 24 h (Table 1 and Figure 1). In comparison, the samples charred at 250°C lost these hair-like appendages in a shorter duration of 6 h (Table 1 and Figure 1), and the preservation of morphological integrity was very low for samples charred at 250°C for different duration. Progressive heating caused increased fragility with dehydration of the grains. The samples charred for 16 h duration at 230°C resemble the ornamentation similar to the archaeological counterpart both in

Figure 1. Photomicrographs of rice grains charred at 230°C for (***a***) 2, (***b***) 4, (***c***) 8, (***d***) 16 and (***e***) 24 h.

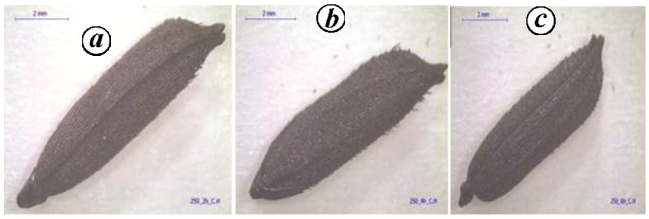


Figure 2. Photomicrographs of rice grains charred at 250°C for (***a***) 2, (***b***) 4 and (***c***) 6 h.

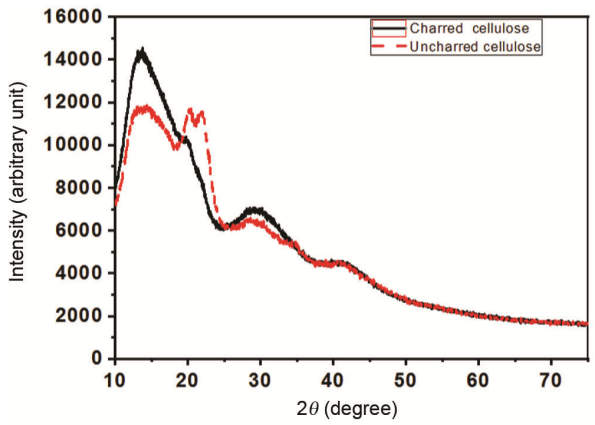


Figure 3. XRD diffraction pattern for extracted cellulose from charred and uncharred rice grains.

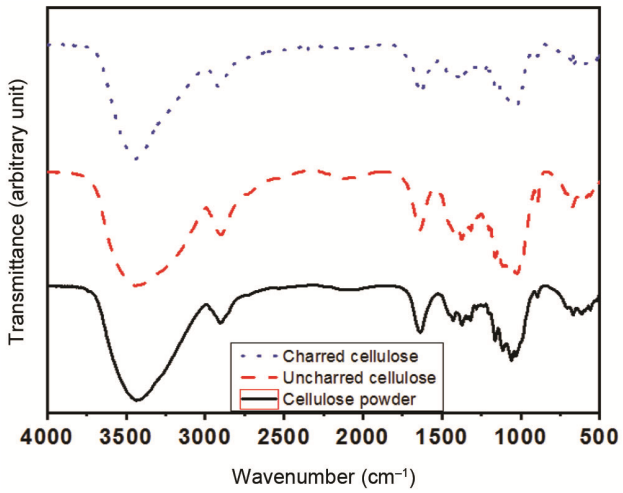


Figure 4. FTIR spectra for extracted cellulose from charred and uncharred rice grains. Also plotted is the spectra obtained for a commercially available pure cellulose powder.

terms of colour and structural morphology. The lack of hair-like appendages observed in archaeological rice grains is probably due to the process of abrasion or friction while the grains are buried. Other features like palea and lemma preserved in the experimental charred grains are similar to archaeological grains retrieved from sites of the Harappan civilization[10]. The morphological transformation recorded in the charred grains is documented in Table 1 and illustrated in Figures 1 and 2.

XRD analysis

XRD analysis of cellulose extracted from the charred and uncharred grains were performed. The XRD peaks of 2θ angle were 15°, 22° and 35°, which match the literature values designated for cellulose[27]. The reduced sharpness of peaks was related to the lower crystallinity and more amorphous nature of the sample (Figure 3).

FTIR analysis

In order to confirm the presence of cellulose in the charred grains, we conducted a separate experiment where FTIR spectra were generated (Figure 4) for cellulose obtained from both the charred and uncharred samples. Sample peaks were observed at around 3440 cm^{-1} and 1640 cm^{-1} for both the charred and uncharred cellulose remains, which is attributed to the stretching of hydrogen bond and a hydroxyl group (OH) associated with cellulose structure. The 3440 cm^{-1} peak is due to C–H and O–H group, and 1640 cm^{-1} peak due to absorption of water. Absorbance at 1056 and 896 cm^{-1} is due to C–O bond stretching and C–H bond vibration of cellulose. All the spectra associated with lignin at 1737, 1507, 1436, 1507 cm^{-1} were absent in both the spectral display[28]. From the above data, presence of cellulose in both charred and uncharred samples was evident. Lignin was found absent in both these samples. This indicates that both the cellulose samples were pure, i.e. free of lignin.

Effect of charring on isotopic composition

Palaeoclimatic reconstruction is possible upon analysis of carbon isotope ratios in the grains of archaeological

Table 2. Stable carbon isotope isotopic composition of caryopsis and husk of rice samples and extracted cellulose

Temperature (°C)	Duration (h)	$\delta^{13}C_{VPDB}$ (‰) caryopsis	$\delta^{13}C_{VPDB}$(‰) husk	$\delta^{13}C_{VPDB}$ (‰) cellulose
Uncharred	–	–28.72	–27.84	–27.49
230	24	–28.35	–28.38	–27.58
250	6	–28.65	–28.43	–

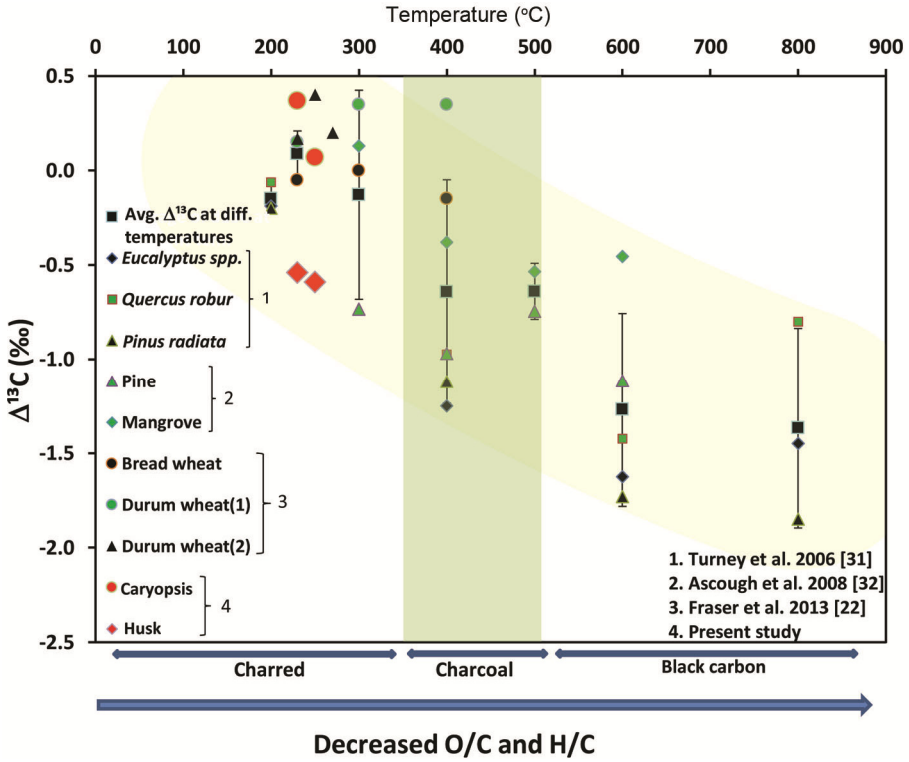


Figure 5. $\Delta^{13}C$ (initial $\delta^{13}C$ – final $\delta^{13}C$) versus temperature (°C) plot. The $\delta^{13}C$ and temperature data from the present study were collated with previous studies[22,31,32]. Error bars represent 1 standard deviation about the average $\Delta^{13}C$ values.

rice[6,10]. Charring being one of the common modes of preservation, it is important to understand how it can alter the original isotope composition of different plant parts such as seed, stem, root, wood, etc. which can give useful information in past environmental reconstruction studies. The systematic offset of carbon isotope signature between the plant remains (charred sample) and original signature, if corrected, would enable estimation of true isotopic composition. Studies show different offset at different temperature of charring. A previous study on charring of peas at a temperature range of 100–700°C caused ~1.5‰ shift in the original $\delta^{13}C$ values[29]. Similarly, Charring of barley and wheat grain at a temperature range of 230°C caused – 0.5‰ and +1.2‰ shift in the original $\delta^{13}C$ values respectively[30,31]. In a study by Fraser *et al.*[22] different preservation methods like burial, charring, humic acid contamination and acid–base–acid treatment were experimented on cereals and pulses to decipher shift in the carbon and nitrogen isotope composition. In this study bread wheat and peas were charred at 230°C for a duration of 2–24 h in a reducing condition. The average in $\delta^{13}C$ values due to charring for different time duration was found to be –0.8‰. The ensemble of data from charring experiment on all different kinds of cereals, pulses and wood were collected in Figure 5, together with observations from the present study. Samples of wood of *Eucalyptus* spp., *Quercus robur* and *Pinus radiata* were subjected to the charring temperature range 200–800°C (ref. 31). Similarly, pine and mangrove wood were also subjected to the temperature range 300–600°C during charring[32]. To examine the effect of time bound shift in isotopic composition at different charring temperatures irrespective of the different protocols used for conducting charring process involving different plant parts, we introduced a normalization scheme where deviation from the original composition at a particular temperature was

estimated and defined as $\Delta^{13}C$ (difference between the original and final $\delta^{13}C$ value). The average $\Delta^{13}C$ values for each charring temperature were calculated and plotted with temperatures as an independent variable. Together with this, the process of pyrolysis is likely to modify the carbon (C), oxygen (O) and hydrogen (H) content in the charred grains; while C content increases, the O and H reduces. This leads to a drop in H/C and O/C ratios with increment in temperature[33], as also observed for charred rice and other plant species plotted in Figure 5. The variation of isotope composition ($\Delta^{13}C$) was found to be higher in case of high temperature pyrolysis, whereas low temperature charring gives lesser variability (Figure 5). For rice husk, the maximum variation of $\Delta^{13}C$ was found to be within –0.6‰ for the experiments conducted at the two different temperatures. The difference in $\delta^{13}C$ of extracted cellulose from charred and uncharred samples was –0.1‰ and the difference between charred and uncharred caryopsis was less than –0.4‰ (Table 2, Figure 5). Thus, the isotopic variation due to the charring process was relatively lesser as compared to the variation observed for other cereals discussed above. Therefore, rice archives from archaeological sites can be used as an independent proxy for deduction of the palaeoclimatic conditions and provide validation to the other contemporary proxy records.

Conclusion

There have been several studies to understand the reliability of $\delta^{13}C$ of organic carbon in deduction of past climatic condition by analysing different cereals, pulses and wood. From the present study, it can be concluded that rice samples from archaeological sites can be used for palaeoclimatic reconstruction. Although rice samples were charred at different temperatures and duration, the retention of morphological features was acceptable until charring duration of 16 h at 230°C. The $\delta^{13}C$ value of cellulose was higher than that observed for husk due to absence of the ^{13}C depleted lignin. The change in $\delta^{13}C$ values after charring were relatively less as compared to that observed for other cereals and were <0.4‰ for caryopsis, within –0.1‰ and –0.6‰ for cellulose and husk, as compared with their original counterpart. The observed trends of $\delta^{13}C$ for husk and caryopsis could be due to the difference in their biochemical constituents. The overall depletion in ^{13}C isotope value for husk could be due to the degradation of cellulose, and enrichment of ^{13}C in caryopsis is due to loss of ^{12}C enriched polysaccharides with prolonged charring. Thus, in order to use a particular type of archaeobotanical sample, we need to have prior knowledge about the extent of thermal alteration at a particular archaeological site due to burial or fire so that minimum alteration of the original isotopic signature is ensured.

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