Morphology, X-ray Diffraction and Solubility of Underutilized Legume Starch Nanocrystals

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Abstract*: **Nanocrystals of the native starches isolated from lima bean (Phaseolus lunatus) and jack bean (Canavalia ensiformis) were prepared by mild acid hydrolysis. The shapes and granular sizes of the native starches were obtained by a scanning electron microscope. Platelets that existed as aggregates rather than discrete molecules were observed from the transmission electron micrographs of the nanocrystals. The X–ray diffraction of the starch nanocrystals showed that they were of V–type crystalline structure. Lima bean nanocrystals were sparingly soluble in acetic acid, ethanol and deionized (DI) water. These starch nanocrystals could be potential precursors to nanocomposites and nanoparticle-based copolymers.***

Keywords: Nanocrystals, native starches, solubility

1. Introduction

Starch, a biocompatible, biodegradable, non-toxic polymer [1], has, in recent times, gained increased attention in food and non-food applications due to a host of advantages, which include low density, cost effectiveness, abundant supply and environmental amity [2]. It is widely used in food, paper-making, fine chemicals, packing materials, pharmaceuticals, rubber and plastic industries [3]. The major botanical and commercial sources of starch are cereals, roots and tubers, and pulses [4]. Other sources, classified as minor, are legumes. In its native form, starch is unusable in some applications. The undesirable behaviours of native starch can be reduced or eliminated through modifications by reorganizing the structural arrangement of the starch granules, resulting in enhanced physicochemical properties.

Starch granules in nanometer range have been shown to have unique and novel functional properties. Nanoparticles of native starch called nano starch or starch nanocrystals can be obtained by acid hydrolysis [5]. Worldwide commercial foods and food supplements containing added nanoparticles are becoming available. A major growth area appears to be the development of “nanoceuticals” and food supplements [6].

The application of nanocrystals of starch has gone beyond food. Interest in biomaterials and nanocomposites has now emerged [5, 7, 8]. The quest for nanocrystals of starch has increased the demand for starch for both food and non-food applications. Thus, high competition arises between the domestic and industrial uses of most commercial starches from normal and waxy corn, rice and potato. It is, therefore, imperative to explore alternative sources of starches to complement the ever-increasing demand in the global market. A good way of doing this is by exploring underutilized crops. Such crops, as found in the tropics, include lima beans and jack beans. They are termed underutilized because of their poor domestic usage.

Lima bean (*Phaseolus lunatus L.*) is a perennial American legume species with annual cultivars. It is widely distributed in Latin America, the southern United States and Canada, and many other regions worldwide. The seeds are a rich source of protein (24%) and starch (63%) [9].

Jack bean (*Canavalia ensiformis*) belongs to the family of the *Leguminosae*. It is native to the West Indies and Central America, but is now found scattered throughout the tropics and sub–tropics [10], having different native names. Presently in Nigeria, there are no farms where jack bean is commercially cultivated. People plant jack bean as a flower around their homes while some grow wild.

The aims of this research work were to (a) isolate the native starches from lima bean (*Phaseolus lunatus L.*) and jack bean (*Canavalia ensiformis*), (b) prepare the nanocrystals of the starches, and (c) investigate the morphology, crystalline nature and solubility of the starch nanocrystals.

2. Literature Survey

Nanotechnology is based on the development of innovative and efficient materials while renewable raw materials focus on sustainable products for the development of future applications [11]. Starch nanocrystals are candidates of growing interest for the production of biobased nanomaterials. They are crystalline platelets resulting from the disruption of the semicrystalline structure of starch granules by the hydrolysis of amorphous parts [11].

The dominant component of the crystalline region in native starch granules is thought to be amylopectin lamellae [12], which pack together to form double helix crystal structure [13]. In crystallites of starch, parallel stranded double helical structure is found in pairs, and all chains are packed in arrays. The pairing of double helices is identical in both polymorphs and corresponds to the interaction between double helices that has the lowest energy [14]. The crystalline regions of starch granules can be isolated by mild acid hydrolysis using hydrochloric or sulfuric acid. It is believed that at temperatures below the gelatinization temperature acid molecules preferentially attack the amorphous regions of the granule [15], resulting in these regions being more rapidly hydrolyzed than the crystalline regions [16]. The residue after acid hydrolysis contains the starch nanocrystals which have nanoscale platelet morphology.

3. Previous Work

The morphology of starch nanocrystals extracted from waxy maize was revealed in 2003 [13]. Transmission electron microscopy (TEM) observations showed a longitudinal view of lamellar fragments consisting of stack of elongated elements, with a thickness of 5–7 nm, and a planar view of individualized platelets after hydrolysis. Attempts have, however been made to prepare nanocrystals from different starch sources [5], [8], [17], [18]. Irrespective of the shape of the nanocrystals, all nanocrystals can be considered as potential fillers in nanocomposites [11]. Studies on corn starch and waxy starch nanocrystals as reinforcing fillers in natural rubber have revealed possible replacement of conventional filler, carbon black with nano starch of corn [17].

Upto now, there is no information yet on attempts on preparation of nanocrystals of legume starches. Hence, there is justification for attempts on preparation of nanocrystals of starches sourced from underutilized lima bean and jack bean.

4. Materials and Methods

4.1 Materials

The seeds of lima bean (*Phaseolus lunatus*) and pigeon pea (*Cajanus cajan*) were purchased at Jattu Market, Edo State, Nigeria while seeds of jack beans (*Canavalia ensiformis*) were harvested from the residential orchards, both in Owo, Ondo State and Auchi, Edo State, Nigeria. All the chemicals used were analytical grades and were used directly without further purification.

4.1.1 Isolation of Native Starch

Starches from the legumes were isolated by adopting the method described by Galvez and Resurreccion [19]. The general scheme used included cleaning the seeds, washing prior to stepping process, steeping, wet-milling and settling procedure to isolate starch from the suspension and sieving, disinfection and drying.

4.1.2 Preparation of Starch Nanocrystals

The acid hydrolysis method described by Angellier *et al.* [8] was adopted. 37 g of native starch granules was mixed with 250 ml of 3.16M H_2SO_4. The suspension was placed over a water-bath at a regulated temperature of 40^oC for 5 days. Continuous stirring was ensured by means of homogenizer set at 100 rpm. After 5 days, suspension was washed by successive centrifugation in distilled water until neutral. The aggregate was freeze-dried at 4^oC with several drops of chloroform.

4.2 Methods

4.2.1 Scanning Electron Microscopy (SEM*)*

The starch samples were sprinkled onto the aluminum specimen stubs with double-sided adhesive tape while the non-sticking portion was blown off. The samples were coated with a 30 nm layer of gold using a sputter coater [Polaron (Fisons) SC 515 VG Microtech, Sussex, UK]. The coated starch samples were observed using a Scanning Electron Microscope (FESEM Leo Supra 50VP, Carl-Zeiss SMT, Oberkochen, Germany). Images were captured at different magnifications of 1000 K X, 2000 K X and 5000 K X for morphological studies.

4.2.2 Transmission Electron Microscopy (TEM)

The suspension of nanocrystals of starch was dispersed in ethanol (100%, v/v) and sonicated for homogeneity of the nanocrystals for 3 mins, using Sonicor (Copiague, NT). A drop of dilute nanocrystal suspension was spread on a glow-discharged copper-coated TEM grid and was allowed to dry for 3 mins. The preparation was negatively stained with 2% (w/v) uranyl acetate, and was allowed to spread for 1 min. The grid holding the stained nanocrystals was placed in a petri-dish for 15 mins to dry. The dry grid was observed using a Philips CM 12 microscope (FEI Company, Eindhoven, Netherlands) operating at 80 kV. Images were recorded on Kodak S0163 film.

4.2.3 X-Ray Diffraction Patterns

The X-ray diffraction studies were carried out using a Siemens D5000 X-ray Powder Diffractometer (20^o Geometry, Madison, USA). The starch nanocrystals were equilibrated with distilled water in a dessicator of 48 h before determination to improve resolution of the X-ray diffractogram pattern. The fine samples were filled into a sample holder and packed as densely as possible. The finished surface was smoother and flushed. The samples were mounted into a X–ray diffractometer and copper Kα, 2λ ($\lambda = 1.540\ \mu$m and 1.544 Å; 40 KV; 35 mA) was generated to determine X-ray pattern. The scan was made from a diffraction angle (2θ) of 1.5 to 70^o at 0.05 step size with a count time of 3 s. From the resulting X-ray patterns, peak positions were identified using the instrument's software and these peak positions were used to determine the crystalline natures of the starch nanocrystals [20].

4.2.4 Solubility Test of Starch Nanocrystals

The method described by Xu *et al.* [18] was adopted for the determination of solubility of starch nanocrystals in organic solvents with some modifications. Five (5) organic solvents and one (1) organic solvent were used. The solvents used were toluene, xylene, acetic acid, trichloromethane (chloroform), ethanol and deionized water. 5 mg/ml concentration of the nanocrystals was prepared each with the solvents at room temperature. The resulting contents were slightly agitated and left undisturbed for about 30 mins after which the contents were observed and photographs taken. The contents were left for 24 h to investigate any change in the solubility. No differences were observed in the solubility of the contents.

4.3 Statistical Analysis

Duncan's least significant test was used to compare means at the 5% significance level. Simple Pearson correlation and regression analysis was conducted using SPSS 17.0 software (SPSS Inc., Chicago, IL).

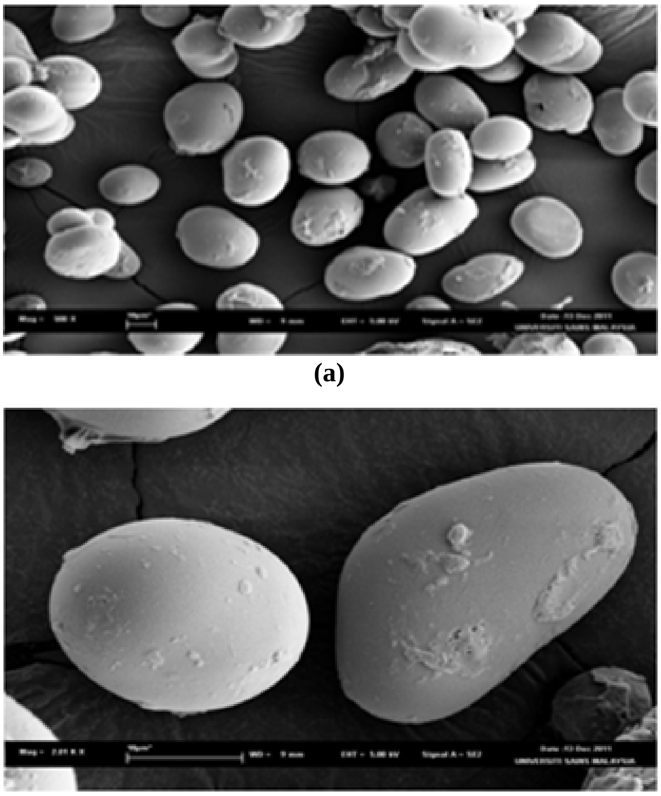
5. Results and Discussion

Table 1 shows the percentage yields of the native starches and their nanocrystals from lima bean and jack bean. The percentage yield of native starch obtained from lima bean was slightly higher than jack bean whereas an opposite trend was observed for the starches in terms of their nanocrystals. The differences in percentage yield of nanocrystals could be, apart from the botanical source, adduced to the molecular interaction among the starch granules during acid hydrolysis.

Table 1: Percentage Yield of Native Starch and Starch Nanocrystals

Starch	*% Yield*	
	Native	*Nanocrystals*
Lima Bean	20.36	10.81
Jack Bean	20.26	13.51

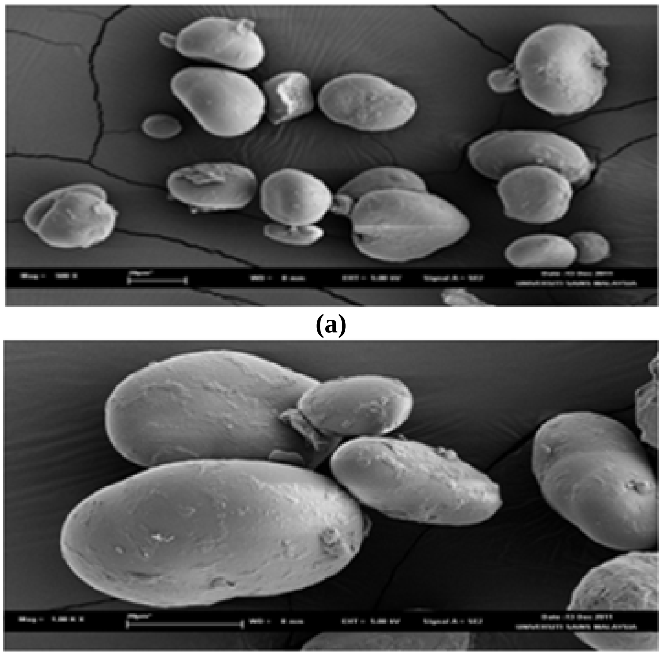
The starch granules of the native starches observed with scanning electron microscope varied in shapes (Figure 1–2). The mean granular sizes of the native starches, measured with the aid of Scanning Electron Microscope (FESEM Leo Supra 50VP, Carl-Zeiss SMT, Oberkochen, Germany) were 23.08 μm and 26.23 μm for lima bean and jack bean starches respectively. The granules of lima bean starch were mainly spherical and oval. Jack bean starch granules were mixtures of oval, ellipsoidal and bean–like granules. Lawal [21] had reported oval or elliptical granular shapes for starch granules of underutilized pigeon pea. The surfaces of the starches were minimally rough. The roughness could not be adduced to damages, but the presence of surface proteins, which can be eliminated by intensive purification process of the starch samples. However, these observations suggested that the processes of extraction and drying had no damaging effect on the starches.



(a)

(b)

Figure 1: Scanning electron micrographs of lima bean starch at: (a) 0.500K ×, 10 μm; (b) 2.00K ×, 10 μm



(a)

(b)

Figure 2: Scanning electron micrographs of jack bean starch at: (a) 0.500K ×, 10 μm; (b) 2.00K ×, 3 μm

The transmission electron micrographs of the nanocrystals are shown in Figure 3–4. Platelets were formed as a result of crystallization of the amylopectin molecules of the starches after mild acid hydrolysis for five (5) days. The platelets were not discrete molecules, but existed in aggregates. These observations were in line with the report of Angellier [22] for waxy corn starch nanocrystals. Lima bean starch nanocrystals were observed to be aggregate of spherical parallelpiped blocks (Figure 3) while jack bean starch nanocrystals appeared as aggregate of lamellar fragments stacked together (Figure 4).

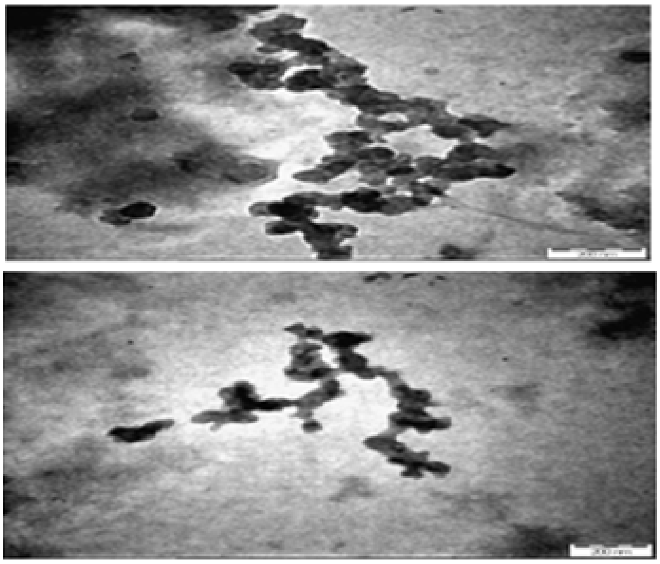


Figure 3: Transmission electron micrographs of negatively stained preparations from lima beans starch granules treated with H_2SO_4 at 40^{O}C under continuous stirring (200 nm)

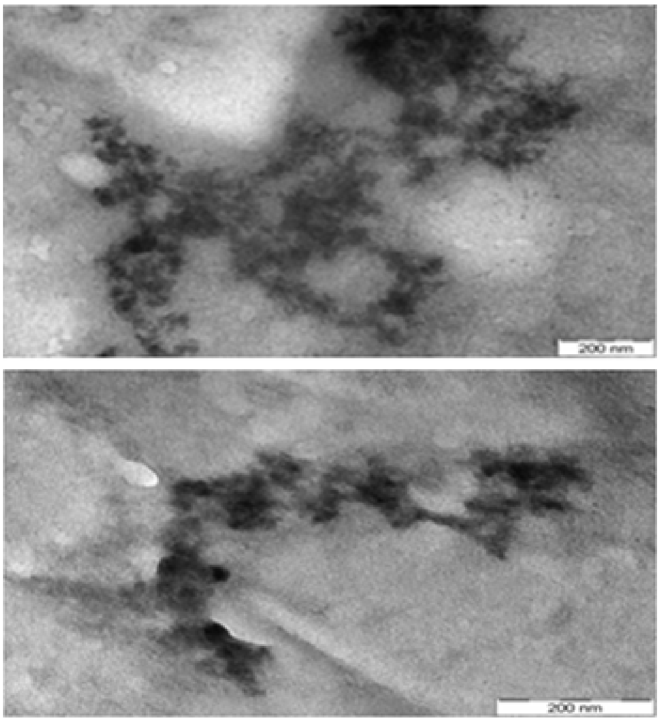
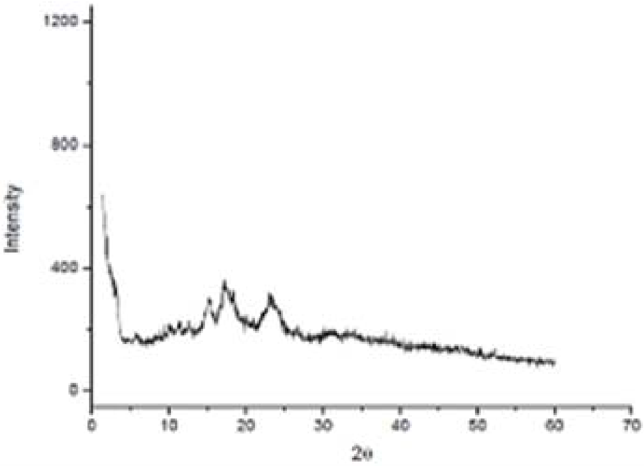


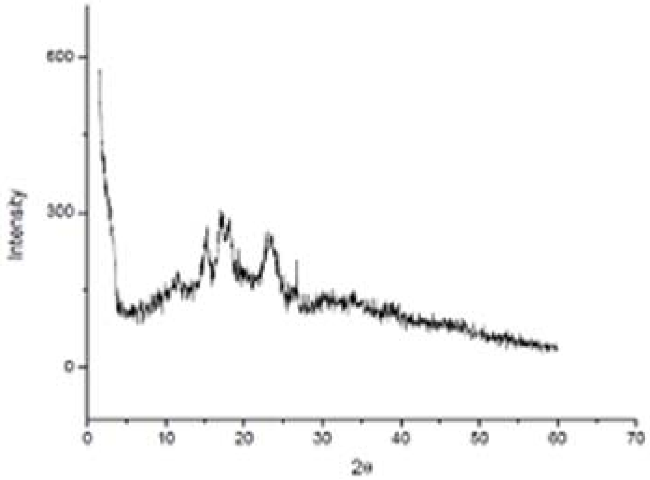
Figure 4: Transmission electron micrographs of negatively stained preparations from jack bean starch granules treated with H_2SO_4 at 40^{O}C under continuous stirring (200 nm)

Putaux *et al.* [13] had reported Transmission electron microscopy (TEM) observations for waxy maize in terms of: (a) a longitudinal view of lamellar fragments, consisting of stack of elongated elements, with a thickness of 5–7 nm, and (b) a planar view of individualized platelets after hydrolysis. They reported that shapes and lateral dimensions were derived from observation of individual platelets in planar view and marked 60–65^{o} acute angles for parallelepiped blocks with a length of 20–40 nm and a width of 15–30 nm. However, more recent publications reported bigger starch nanocrystals [23], [24], with round edges [25] and found as grape-like aggregates of 1–5 μm. The aggregate nature of starch nanocrystals has been adduced to hydrogen bond interaction via the surface hydroxyl groups [18]. This implied that the particle sizes of the individual platelets were not altered by the platelet–like, as aggregate, of the nanocrystals. Irrespective of the shape of the nanocrystals, it has been reported that all nanocrystals can be considered as potential fillers in nanocomposites [11]. This, therefore, suggests that all the starch nanocrystals prepared in this study are potential fillers in nanocomposites. Starch nanocrystals obtained by acid hydrolysis of potato and waxy maize starch granules have been used as filler in a synthetic polymeric matrix and appeared to be an interesting reinforcing agent in natural rubber [17], polylactic acid, and polycaprolactone for getting nanocomposites with unique properties [26] and pharmaceuticals. Starch nanocrystals can, henceforth, serve as replacement for the use of petrochemicals in the food and non–food applications.

X–ray diffraction peaks for the native starches appeared at 15.20^{o}, 17.20^{o} and 22.90^{o} 2θ for lima bean, corresponding to interplanar d–spacing of 5.76 Å, 5.15 Å and 3.87 Å and 15.25^{o}, 17.00^{o} and 22.75^{o} 2θ for jack bean, corresponding to interplanar d–spacing of 5.80 Å, 5.12 Å and 3.91 Å (Figure 5). The crystals of lima bean and jack bean were mixes of A– and B–polymorphs with B–polymorph more predominant. Hence, they were classified as C_B–type of crystallinity. These observations were in line with previous reports for legume starches [4, 28]. The The X-ray diffractograms of the starch nanocrystals showed no significant peaks (Figure 6).

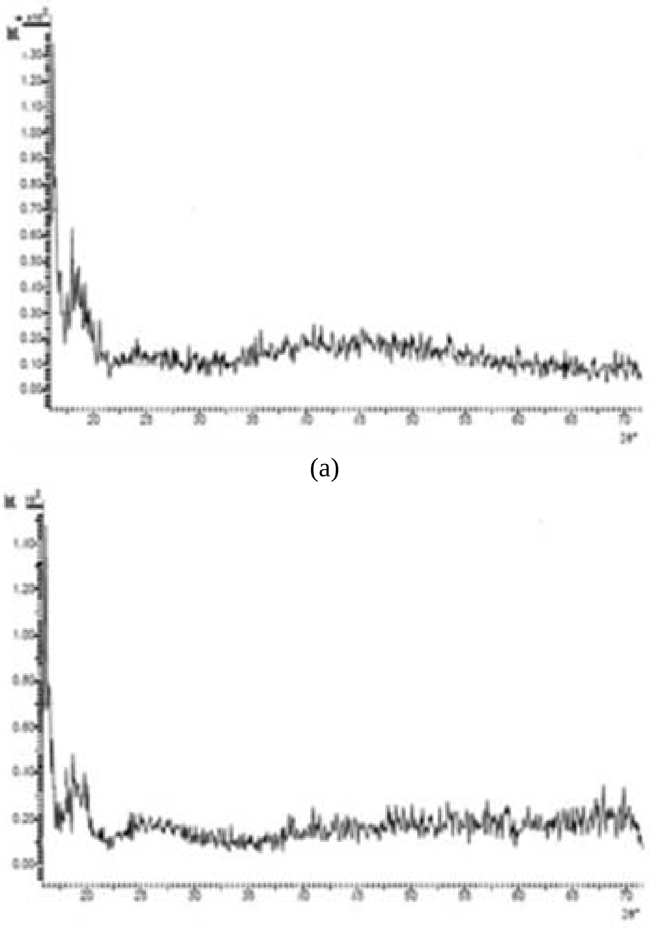


(a)



(b)

Figure 5: X-ray patterns of native starches: (a) lima bean, (b) jack bean



(a)

(b)

Figure 6: X-ray patterns of nanocrystals of: (a) lima bean, (b) jack bean

However, weak peaks appeared at $2\theta \approx 16^o$ (lima bean) and $17^o/20^o$ (jack bean). The possibility for these observations could be that mild acid hydrolysis, by which starch nanocrystals were produced, resulted in the formation of amylose–lipids complex in the amorphous lamella. This could make the amorphous regions larger than the crystalline region. The V–pattern is relatively amorphous with a few weak lines that show crystallinity [28]. It is opined that mild acid hydrolysis altered the crystallinity nature of these starches from C_B–type to V–type and that the relative amorphousity of the starch nanocrystals was not as a result of its high water content. This assertion is true, based on the fact that X–ray powder diffraction is usually done on hydrated starch samples [4]. Hydration is accomplished by equilibrating the sample in a desiccator maintained at a certain relative humidity and temperature.

The results of solubility test of starch nanocrystals with six different solvents (both organic and inorganic), namely toluene, xylene, trichloromethane (chloroform), acetic acid, ethanol and de–ionized water are presented in Table 2. The starch nanocrystals were practically insoluble in toluene and xylene. Their dispersion in trichloromethane resulted in the formation of precipitates, which appeared less dense than the solvent. For lima bean starch nanocrystals, the precipitates floated and appeared as large aggregate (black blocs) whereas for jack bean starch nanocrystals, they floated and appeared as aggregate in fragments. In acetic acid and ethanol, the starch nanocrystals of lima bean appeared sparingly soluble with cloudy supernatant.

Table 2: Solubility Test of Nanocrystals of Starches

Solvent	*Starch*	
	Lima Bean	*Jack Bean*
Toluene	Insoluble	Insoluble
Xylene	Insoluble	Insoluble
Chloroform	Precipitate formed	Precipitate formed
Acetic Acid	Sparingly soluble	Insoluble
Ethanol	Sparingly soluble	Insoluble
De-ionized Water	Sparingly soluble	Suspension formed

The nanocrystals of lima bean appeared as sparing solubles in de-ionized (DI) water. The nonpolarity of chloroform coupled with its linear chemical structure could be adduced to its precipitating ability, when used to disperse starch nanocrystals. DI water has better performance as inorganic and polar solvent due to its low concentration of ions. Xu *et al.* [18] had reported insoluble dispersion for starch nanocrystals of waxy corn with some organic solvents, except dimethylsulphoxide. Dispersion of starch nanocrystals as aqueous suspensions prior to their incorporation into nanocomposite matrix has been a challenge. This is due to its poor solubility in organic solvents, and this singular obstacle, has limited the application of starch nanocrystals as a reinforcing phase in a wide variety of polymers. The use of surfactant to disperse starch nanocrystals in a nonpolar solvent for cellulose whiskers has been proposed [29], but this ends up with large volume of the surfactant being used to maintain the stability of the suspension [18].

6. Conclusion

Nanocrystals of native starches extracted from underutilized lima bean and jack bean have been successfully prepared through mild acid hydrolysis with considerable percentage yield. The micrographs obtained from transmission electron microscope have revealed the nanocrystals as aggregates. Mild acid hydrolysis has been shown to alter the crystalline nature of the native starches from C_B–type to V–type. Lima bean starch nanocrystals appear to be more soluble than jack bean starch nanocrystals. The evidence of these nanocrystals, among other things, is a step towards meeting the quest for nanotechnology in all fields.

7. Future Scope

Further research work should focus on the preparation of modified forms of these nanocrystals and their characterization in terms of FTIR spectroscopy, differential scanning calorimetry (DSC) and molecular mass distribution to elucidate their intrinsic properties for industrial applications. In addition, composite polymeric materials such as hydrogels can be prepared with these nanocrystals.

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