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Characterization by spectrometric methods of chitin produced from white shrimp shells of *Parapenaeus longirostris* by*Lactobacillus helveticus* cultivated on glucose or date waste

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ABSTRACT

Abstract: *Chitin recovery by lactic acid fermentation was considered. It was previously shown that the use of glucose led to 61% of demineralization and 42% of deproteinization; while and unlike glucose, the inoculation of L. helveticus in date waste (200 g L^{-1} reducing sugars) led to a simultaneous demineralization and deproteinization of shrimp shells, 83 and 61 %, respectively. The purity of end-products were characterized by infrared (FTIR), X-ray diffraction and X-ray fluorescence.*

Analysis of the chemical composition of fermentation end-product by X-ray fluorescence showed that residual minerals are in trace except CaO and P_2O_5. The infrared spectra and the X-ray diffractograms of both samples compared to those of pure chitin showed a strong similarity. FTIR of fermented shells showed the appearance of characteristic peaks of α-chitin and the disappearance of calcite peaks. In addition and especially for the shells fermented using L. helveticus cultivated on date waste, a similarity with those of pure chitin should be noted. These results confirmed the efficiency of biological chitin recovery, and that a synthetic carbon source (glucose) can be replaced by a natural one, date waste, allowing a valorization of this latter. The relevance of subsequent works to improve yields of demineralization and deproteinization was therefore confirmed.

I. Introduction

Chitin is a major component in the supporting tissues of organisms such as crustaceans, fungi and insects. Next to cellulose, it is the most abundant biopolymer found in nature [1]. Chitin is structurally similar to cellulose [2,3] In the Exoskeletons of crustaceans, chitin is intimately associated with Other Materials Such as proteins, lipids, calcium carbonate and pigments [3]. Natural, non toxic, biopolymer chitin is now widely produced commercially from crab and shrimp waste shells [4].

Because of their linear (1,4)-β-N-acetylglycosaminoglycan structure with two hydroxyl groups and an acetamide group (Figure 1), native chitin in crustacean shells are highly crystalline with strong hydrogen bonding, and are arranged as α-chitin microfibrils in an antiparallel fashion [5].

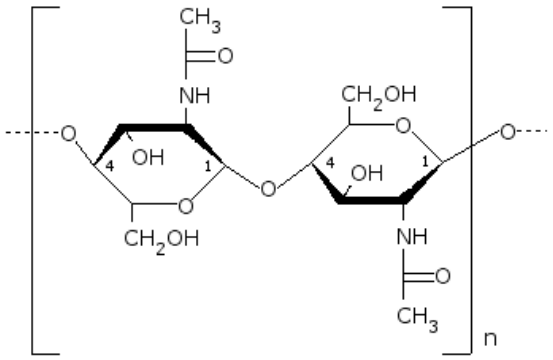

Figure 1.*Chemical structure of chitin*

X-ray diffraction studies reveal that chitin occurs in three polymorphic forms, α-, β-, and γ-chitins, which differ in the arrangement of molecular chains within the crystal cell [6, 7, 8]. α-chitin showing parallel chain packing is the most abundant chitin in nature (shrimps, crabs), β-chitin presents antiparallel chain and occurs in squid pens [9, 10, 11], while γ chitin presents a mixture of α and β chitins [7]. Because chitin possesses many beneficially biological properties such as biocompatibility, biodegradability, hemostatic activity, and wound healing property, much attention has been paid to its biomedical applications [12]. Its main derivative, chitosan, has been used as a dietary supplement due to its effective lipid binding and hypocholesterolemic properties and as a film-forming agent [13]. Chitin, chitosan and their derivatives can be used as antimicrobial, emulsifying, thickening and stabilizing agents in food industry [13].

Recently, biological processes for chitin production have been reported using organic acids producing bacteria [11] and enzymes [14] for the bio-demineralization and the bio-deproteinization of crustacean shells. Lactic acid fermentation of shrimp (*Penaeusmonodon*) waste for chitin recovery was studied with added carbohydrate such as lactose or cassava extract as a natural energy source [15] and date waste for extraction of chitin from head and shell of shrimp *Parapenaeuslongirostris*using *lactobacillus helveticus* [16]. Raw heads of Africa river prawn (*Macrobrachiumvollenhovenii*) were fermented with *Lactobacillus plantarum* using cane molasses [17]. Lactic acid fermentation for demineralization has been also reported for shrimp waste [18], crayfish exoskeleton [19], scampi waste [20] and prawn waste.

In this study, chitin was extracted from shrimp *(Pararenaeuslongirostris)* shells by lactic acid fermentation using *Lactobacillus helveticus* cultivated on glucose or date waste. The biological extraction of chitin requires two main steps bio-deproteinization and bio-demineralization. The calcium and the protein in the shell waste were mainly dissolved by organic acids and proteases produced by microorganisms. The Efficiency of fermentation depends on factors such as the shell content [21].

The aim of this work was to study the efficiency of bio-demineralization and bio-deproteinization. The products were characterized by spectrometric methods (IR, XRD, X-ray fluorescence). Infrared spectroscopy (FTIR) has been proposed as the quickest and most effective way to compare the properties of chitin from different sources (animal and fungal). While the XRD and X-ray fluorescence are used to determine the crystal structure [22] and the quantity of components, respectively.

II. Materials and methods

II.1.Biological Material

This work was carried out using the biological material cited in table 1.

Crude shells, demineralized and deproteinized shells fermented on glucose and datte's juice were harvested [16,23] (table 1) and analyzed in order to study the difference between end-products of each step. Analysis allowed comparing the appearance and disappearance of peaks characteristics of some elements such as protein and calcite.

II.2.Infrared spectra (IR)

To spectroscopically characterize the biopolymer contained in the samples, FTIR measurements were performed and the IR spectra were recorded on a Bruker IFS113vFTIR-spectrometer (Bruker AXS S.A.S., Champs-sur-Marne, France), in the 4000–500 cm^{-1} spectral region at a 2 cm^{-1} resolution. The samples were ground, mixed with KBr powder and then compressed into pellets.

II.3. X-ray diffraction (XRD)

X-ray diffraction measurements were applied to determine the crystallinity of the raw shell, chitin and the end-products. Their patterns were recorded using XRD analysis by means of a monochromatic Cu Ka Philips X'Pert Pro diffractometer (PANalytical, Limeil-Brévannes, France). The 2θ angle was scanned between 5° and 40° and the counting time was 15 s at each angle step. The voltage was 40 Kv and the intensity 50 mA. The intensities of the peaks at (110) lattice (I_{110}, at 2θ~20° corresponding to the maximum intensity)

and at 2θ~16° (amorphous diffraction) were used to calculate the cristallinity indice I_{CR} following equation [24]:

$$I_{CR} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad \textbf{(1)}$$

II.4. X-ray fluorescence

X-ray fluorescence was carried using Magix Pro (PANalytical, Limeil-Brévannes, France) x-ray spectrometer for crude and fermented shells to identify the elemental composition of the different minerals present in shrimp shell before and after lactic acid fermentation.

Table 1.*Characterization of the biological materialused in this work*

	Crude shell		**Fermented shell**			
	[16] 1	[23] 2	[16]	[16]	[23]	[23]
			DP[a] 80 g L^{-1} glucose 30°C	DM[b] 300 g L^{-1} glucose 35°C	DP and DM[c] 300 g L^{-1} glucose 30°C	DP and DM[c] 200 g L^{-1} reducing sugars 30°C
pH	8-9	8-9	76 % DP	20.6% DP	42.14% DP	83% DP
Humidity (%)	3.25	3.25	0% DM	53% DM	61% DM	61% DM
Ashs (%)	**35.5**	**27**				
Proteins (%)	**27.61**	**12**				
Chitin (%)	23.61	23				
Pigments and lipids (%)	13.28	35				

[a]*Deproteinized shell*
[b]*Demineralized shell*
[c]*Simultaneous demineralization and deproteinization*

III. Results and Discussion

Characteristics of shells

The physico-chemical analysis of powder shells was based on the determination of protein content, minerals, chitin and water content. The obtained results are summarized in Table 1. It can be observed that for the same species, shell composition displayed variations. The first sample of crude shells (Table 1) shows a strong mineralization (35.5%) and significant protein content (27%); while the second crude sample had a low mineral level (27%) and a low protein content (12%). Infrared spectroscopy is a technique, which has the advantage of inexpensive method for identification of chemical groups such as (NH, CO) present on the surface of raw material. The spectra of the two shells are shown in figure 2.

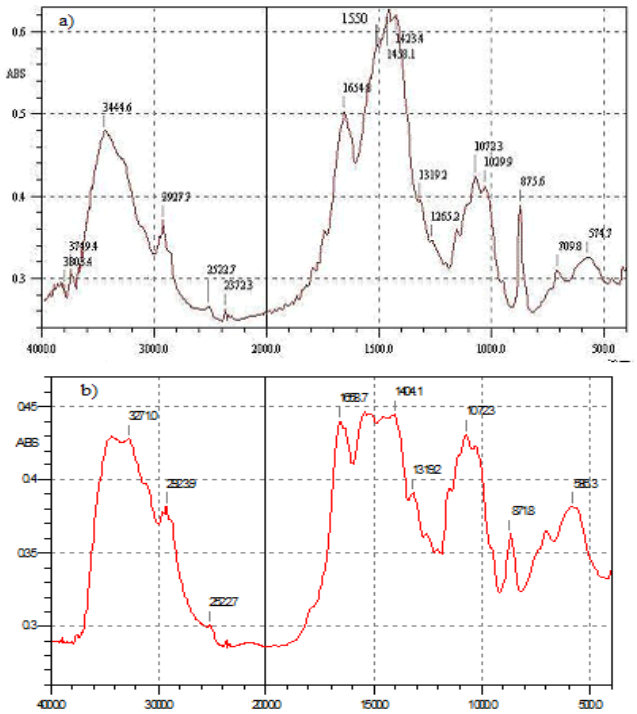

Figure 2.*Infrared spectra of raw shell (sample 1 – Table 1) (a) and (sample 2 – Table 1) (b)*

The infrared spectrum (a) shows that in addition of characteristics peaks of calcite (871 cm^{-1}, 1404cm^{-1}, 2522cm^{-1}), there are characteristic peaks of α-

chitin (1658cm $^{-1,}$ 1319cm $^{-1,}$ 1072cm $^{-1,}$ 586cm $^{-1}$) (Fig. 2).

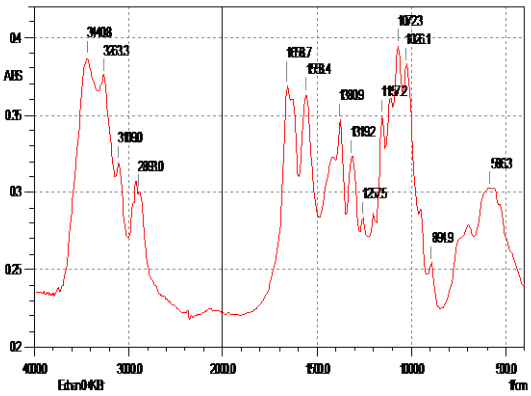

Figure 3. *Infrared spectrum of pure chitin*

While the spectrum of the first sample of raw shell shows the appearance of a peak characteristic of chitin (COC) stretching at 1072 cm $^{-1}$ and a strong band at 1654cm^{-1}, which corresponds to the (C = O) stretching, characteristic of amide I band and peak at 1550 cm^{-1} corresponding to (NH) stretching characteristic of amide II. This difference may be attributed to the high rate of minerals and proteins present in the the first sample of raw shell.

Comparing with the spectrum of pure chitin (Fig.3), figure 1, shows the absence of calcite peaks and the presence of other intense absorption peaks at 1157 cm $^{-}$1, 1380 cm $^{-1}$, 1558 cm $^{-1}$, 2893 cm $^{-1}$, 3109 cm $^{-1}$ and 3263.3 cm $^{-1}$ which are characteristic of α-chitin [25, 26, 27, 28].

Analysis of end-products after lactic acid fermentation of the first sample of shrimp shells using *Lactobacillus helveticus* cultivated on 300g L^{-1} glucose at 35°C (53% demineralization) and on 80 g L^{-1} glucose at 30°C (76% deproteinization) [16] gave infrared spectra shown in figure 4.

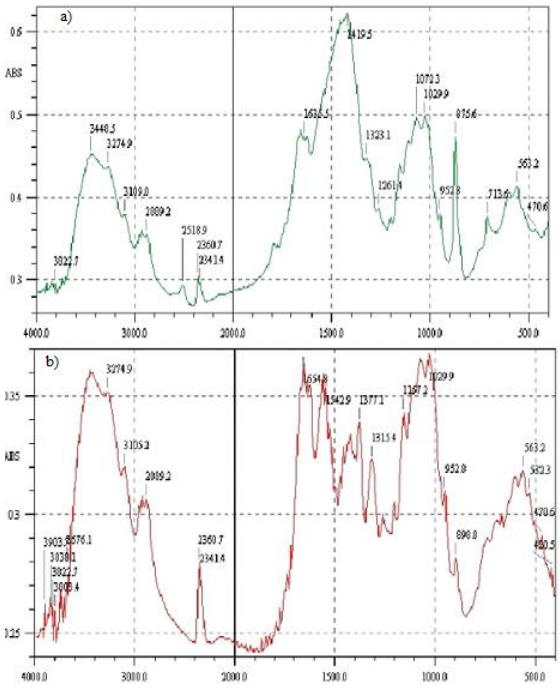

Figure 4. *Infrared spectra of deproteinized (76% with 80g L^{-1} glucose at 30°C) (a) and demineralized (53% with 300g L^{-1} glucose at 35°C) (b) shrimp shells*

The persistence of characteristic bands of calcite in the spectrum of deproteinized shells (Fig.4a) can be noted. New peaks at 2893 cm^{-1}, 3109 cm^{-1} which correspond to (CH) stretching also appeared, which are characteristic of α-chitin [29]. Contrarily, the spectrum displayed in Figure 4b shows a disappearance of bands characteristic of calcite, with appearance of intense characteristic bands of α-chitin at 2893 cm^{-1} and 3109 cm^{-1} (CH) which have been masked by calcite bands [29].

In order to improve the obtained results, another round of extraction of chitin by *Lactobacillus helveticus*, cultivated on glucose or date waste was carried out [23]. The physicochemical analysis of the raw shell showed lower protein content (Table 1). The use of glucose, with initial concentration of 300g L^{-1} at 35°C, as fermentation medium for chitin recovery led to 61 and 42% of demineralization and deproteinization, respectively; while, the use of date waste corresponding to 200g L^{-1} of reducing sugars at 30°C led to simultaneous efficient DM and DP, 61 and 83% [23]. The obtained products were characterized by infrared spectroscopy. The spectra obtained are shown in Figure 5.

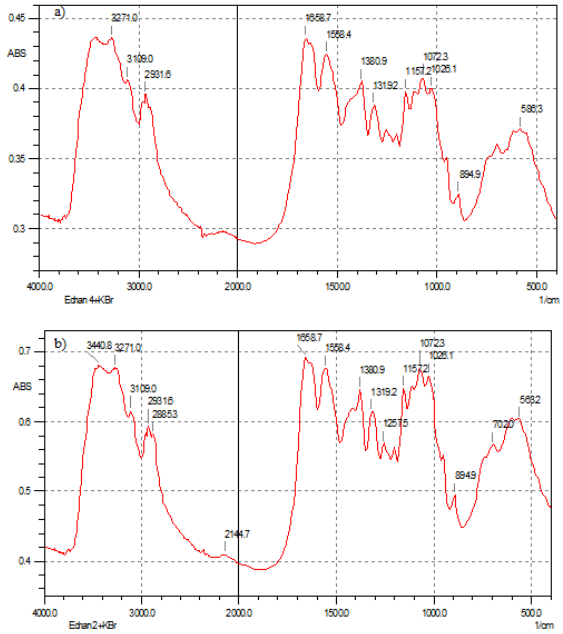

Figure 5. *Infrared spectra of fermented shell (a) using L. helveticus growing on date waste (200g L^{-1} reducing sugars at 30°C) (a) and on glucose (300g L^{-1} at 35°C) (b).*

Infrared spectra of the fermented shells with glucose (Fig.5a) and juice date (Fig.5b) show the disappearance of characteristic calcite peaks, and intense bands were found at 1658 and 1558 cm^{-1}. Both are typical for amides and corresponded to stretching vibrations of carbonyl groups, including in chitins (C=O usually called amide I) and to NH (amide II) respectively [29]. In addition, the spectra were lacking a signal at 1540 cm^{-1}, where proteins normally would give rise to absorption [3] and as well as the appearance of new peaks corresponding to α-chitin.

The determination of the different absorption peaks and the different bands characteristics of chemical groups, observed in each sample, show a significant similarity between pure chitin and fermented shells (Table 2).

Table 2. *The characteristic peaks of the samples [25, 26, 27, 28]*

pure α-chitin	Fermented shell (with glucose 300g L^{-1} at 35 °C)	fermented shell (with juice date 200g L^{-1} reducing sugars at 30 °C)	crudeshell	The chemicalgroups
-	563	-	-	
586	-	586	586	
-	702	-	-	COC stretching
-	-	-	871	calcite
894	894	894	-	COC bridge (glucosidic linkage)
1026	1026	1026	-	COC stretching
1072	1072	1072	1072	COC stretching
1157	1157	1157	-	Glucose linkage / COC bridge [asymmetric (antisymmetric) stretching] CH stretching
1257	1257	-	-	Amide III band
1319	1319	1319	1319	CH stretching
1380	1380	1380	-	Very strong CO stretch $CaCO_3$ [25]
-	-	-	1404	Amide II band
1558	1558	1558	-	C = O-NH-CH_3 stretching (amide I, α-chitin)
1658	1658	1658	1658	Calcite
-	-	-	2522	CH = C + C elongationelongation (Bertrand, 2006)
-	2144	-	-	CH stretching
-	2885	-	-	CH stretching CH stretching

2893	-	-	-	Very strong CH stretching
-	-	-	2923	Amide Band II
-	2931	2931	-	Intermolecular C ($2_{1)}$ NH ... O = C
3109	3109	3109	-	($7_{3)}$ hydrogen bonds
3263	-	-	-	NH stretching
-	3271	3217	3271	Intermolecular OH ... .3 CH_2 OH and hydrogen bonds5)
3440	3440	-	-	

From Table 2, it can be concluded that the characteristic peaks of α-chitin appeared close to those observed after fermentation using *L. helveticus* growing on date waste.

The diffraction pattern of crude shrimp shell (Fig.6), shows the presence of two intense peaks 2θ = 19.39° and 2θ = 29.53° which was typical of the calcium carbonate present in the carapace (5,30), as well as a low peak at 2θ = 9.16°.

In the case of shrimp material, six signals were identified: at 2θ= 9.2°, 2θ= 12.55°, 2θ= 19.15°, 2θ= 20.8°, 2θ= 22.95° and 2θ= 26.15° [30].

Zhou et al. [26] showed that α-chitin has 2θ diffraction peaks at 9.3°, 19.4° and 26.3 °. The work carried out by Stawski et al. [28] showed that for α-chitin, the maximum were located at 2θ= 20.5° and 2θ= 40.5°.

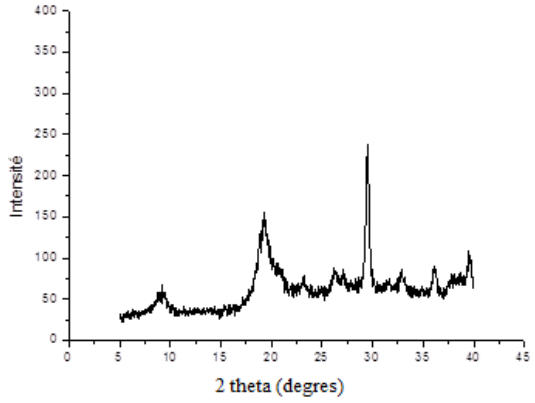

Figure 6. *Spectrum of X-ray diffraction of raw shell*

The characteristic peaks of pure chitin can be observed in both samples, namely obtained after shrimp shell treatment using *L. helveticus*growing on glucose (Fig.7a) or date waste (Fig.7b).

The interesting results obtained using the shell harvested in 2008 compared to those obtained using the shell harvested in 2005, pushed us to analyze by X-ray diffraction and X-ray fluorescence, onlytheend products of fermentation of the shell harvested in 2008.

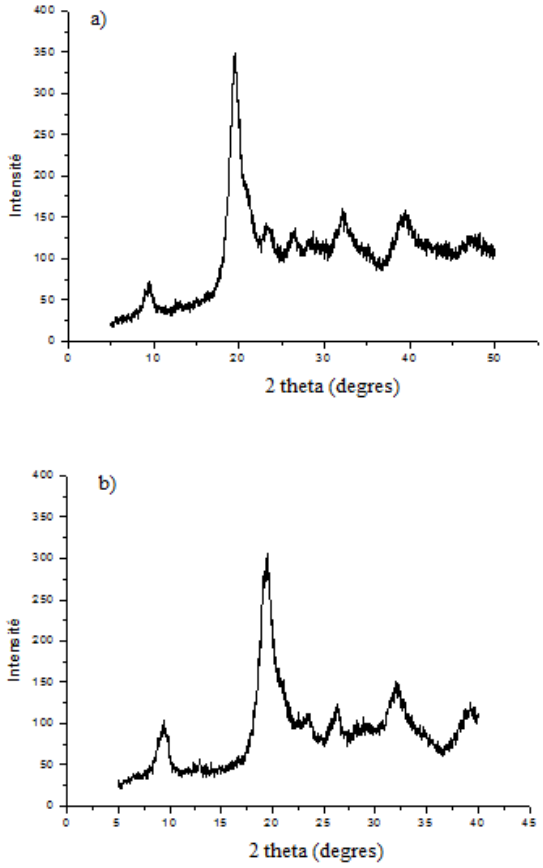

Figure 7. *Spectrum of X-ray diffraction of the carapace treated by L. helveticus cultivated on glucose (a) or the date waste (b)*

The X-ray diffractograms of both samples chitin (glucose and date waste) (Fig.7) were largely similar to α-chitin difractograms described in the literature.

It was found that I_{CR} of the chitin from crab shells and rice-field crab shells (Table 3) were slightly higher than that of the chitin recovered in the present study. This difference may be attributed to the effect of interactions between chitin and impurities explained by the relatively low rates of demineralization and deproteinization.

The chemical composition of the raw shell and the two products previously recovered [23] was determined by X-ray fluorescence. The obtained results are collected in Table 4, and showedthat the majority of minerals present in the raw shell were

Table 3. *I_{CR} values of different samples of chitin*

Sample	$I_{CR\ (\%)}$	2θ (°)
Chitin (glucose)	77.15	9.30°, 19.45°, 23.35°, 26.42°, 32.33°, 39.41°
Chitin (date waste)	72.18	9.55°, 19.49°, 23.45°, 26.25°, 32.14°, 39.39°
α-chitin (from crab shells) [32]	85.0	
chitin from Cicada sloughs (insect) [24]	89.7	
Rice-field crab shells [24]		
Brown shrimp chitin (*Penaeusaztecus*) [33]	91.7	
Pink shrimp chitin (*Penaeusdurarum*)	64	
	66.6	

removed, since they were at trace levels in the fermented shells except for calcium oxide CaO and phosphate P_2O_5.This elimination is due to the production of lactic acid by lactobacilli. This production caused acidification and lowering of the pH to around 4.7 [23] which caused the solubilization of the minerals initially present in in the raw shell and consequentlydemineralization. While the proteolytic enzymes secreted in the fermentative medium by the lactic acid bacterium ensure its deproteinization.

Table 4. *Chemical composition of raw and fermented shells*

Compounds	Raw shell	Fermented shell	
		on 200 g L^{-1} date juice at 30°C	on 300 g L^{-1} glucose at 35°C
Na_2O	1.899	0.004	0.018
MgO	1.739	0.014	0.022
Al_2O_3	0.233	0.037	0.045
SiO_2	2.202	0.04	0.171
P_2O_5	5.323	5.96	5.42
CaO	21.636	9.014	8.9

IV. Conclusion

In order to check for the nature of the residual minerals, the elemental composition of the collected samples was analyzed. The X-ray fluorescence results showed that most elements were in trace. This analysis shows the elimination of 50% calcium oxide initially present in the carapace, which proved the efficiency of the biological treatment.
FTIR of fermented shells showed the appearance of characteristic peaks of α-chitin, and the disappearance of calcite peaks. In addition and especially for the shells fermented using L. helveticus cultivated on date waste, a similarity with those of pure chitin should be noted.
Finally and according to X-ray diffractograms of chitins obtained after biological treatment of shells, the appearance of three major characteristic peaks of α-chitin should be noted, with however some impurities due to residual minerals.
The above results allowed confirming that a natural one, date waste, which also constituted a valorization of this waste, can replace a synthetic carbon source (glucose).

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