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Kinetic study of bio-demineralization and bio-deproteinization of shrimp biowaste for chitin recovery

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ABSTRACT/RESUME

Abstract: *Demineralization (DM) and deproteinization (DP) of shrimp shell Parapenaeus longirostris using Lactobacillus helveticus, depends on the composition of culture medium, and temperature of incubation. In a synthetic medium containing glucose, completely different conditions are required for chitin recovery: 300 g/L of glucose and 35°C for maximal DM (60%) and 80 g/L of glucose and 30°C for a maximum of DP (70%). The use of date's juice shows that it is possible to extract chitin, in a single step, unlike the simple medium. The richness of the dates juice in mineral elements and the high concentration of reducing sugars (200 g/L) allowed for a maximum activity of proteolytic enzymes favored by a pH maintained constant at around pH 6 for 29h of fermentation, and a significant acidification expressed by a minimum pH of 4.7 which are reflected by rates of 83% and 63% of deproteinization and demineralization, respectively.*

I. Introduction

Industrial processes always accompany wastes that pose significant risk to the environment. Technologies that would treat these wastes, or even better recover some useful organic materials before disposal, are necessary to mitigate pollution. Shrimp shells normally contain about 17% of chitin and 42% of proteins; recovery of these useful products or their derivatives is significant from an industrial, for production of enzymes and bioactive materials [1], and ecological viewpoint. Chitin [α-1,4-poly(N-acetyl-D-glucosamine)] can be converted to glucosamine by deacetylation and hydrolysis, while proteins can be hydrolyzed to form amino acids [2].
Chitin can be found in the biosphere; it is the major component of cuticles of insects, fungal cell walls, yeast, green algae [3, 4] and archaea [5]. It is more abundant than any other natural biopolymers except cellulose. The production, properties, and application of chitin and its derivatives have attracted worldwide attention [6, 7]. The potentials of using chitin and chitosan in water treatment, agricultural and food processing [8, 9], cosmetics [10], pharmaceuticals [11], and biotechnology [12] have been extensively investigated for years. To date, the major source of industrial chitin comes from crustacean shells. Seafood processing and consumption generate thousand tons of shellfish wastes each year [6].
The isolation of chitin involves demineralization and deproteinization [13, 6] of shellfish waste with the use of strong acids or bases [14, 15].
Chemical chitin purification is extremely hazardous, energy consuming and damaging to the environment by high concentrations of mineralic acid and caustic [16]. Another disadvantage of chemical chitin purification is a certain degree of depolymerization due to the harsh conditions, and the valuable protein components can no longer be used as animal feed [17]. Recent studies have revealed notable variability in the dye, water, and fat binding capacities of various chitins, chitosans,

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and their derivatives prepared at lab-scale from crustacean shell wastes [18], as well as notable variability in the antibacterial activities [19], the biodegradability and the immunological activities [20, 21]. One possibility of a gentler manufacturing of chitin is the use of proteases for deproteinization of crustacean shells [22], which would avoid alkali treatment. Besides the application of exoenzymes, proteolytic bacteria were used for deproteinization. Alternatively, ensilation of crustacean shells and an in situ lactic acid production by lactic bacteria induced a liquefaction of the semi-solid waste and led to a low pH and activation of proteases [17]. The protein-rich liquor could be separated from the chitin, which remained in the sediment. A side effect of ensilation is the lactic acid production by fermentation of carbohydrates, a concomitant demineralization of the shells and a partial precipitation of the calcium ions as calcium lactate [23].

In several arid countries an over-production of dates exists. The estimated world over-production of dates is 1.2 million tons per an [24]. Algeria produces more than 400 different varieties of dates with an annual production of over 400,000 tons. However, about 20% of the production is lost due to over-ripening and improper handling, processing, and marketing. Dates are rich in carbohydrates: they contain between 60 and 70% by weight of easily extractable sugars, predominantly glucose and fructose. A range of minerals and vitamins [25] are present, and protein content (1.5–3% (w/w)) is low. Utilization of poor-quality dates and date by-products has been studied for bakery and ice-cream, for the production of caramel color, alcohol, vinegar, citric acid, oxytetracycline, as well as for thermophilic dairy starter and single-cell protein preparation [25]. However, the development of a fermentative lactic acid process for using date waste remains attractive from both the preservation of environment and production [25].

In previous work, reference [26] showed that, for an initial pH of 8.5–9.0 and a temperature of 30°C, maximum deproteinization and demineralization were 76 and 53%, achieved for 80 and 300 g/l of glucose, respectively. The level of demineralization increased to 60% for an increase in temperature from 30 to 35°C, the rate of deproteinization is decreased. The use of date's juice, as an alternative to the use of a simple carbon source such as glucose, led at best to 44% of demineralization, for 208 g/L of total sugar at 35°C, and 91% of deproteinization for 80 g/l of total sugars content at 30°C [26].

The aim of this study is to ameliorate the conditions of fermentation (nature and initial concentration of carbon source, incubation temperature) obtained in the previously work realized by reference [26]. Find simultaneous conditions of biodemineralization and biodeproteinization of white shrimp shell; and valorization of two sub-products: shrimp shells white *Parapenaeus longirostris* caught on the Algerian coast and dates "Deglet Nour" cultivated in Algerian south. The choice of these products lies, in the richness of shrimp shell in chitin (23%) and high concentrations of reducing sugars and presence of metal ions necessary for the growth of the lactic acid bacterium *Lactobacillus helveticus*, in the date's juice.

II. Materials and methods

II.1. Shells Preparation

Before use, the flesh, antennas and legs were removed from the shrimp shells. They were then boiled in water for 1 h to remove the maximum amount of flesh. Thereafter, they were dried at 163°C for 1 h in a drying oven. After cooling, the shells were subjected to a thermal shock to facilitate crushing. Using a coffee mill, the shells were crushed to obtain a particle grain size ranging between 1 and 3 mm.

II.2. Date's juice preparation

Dates were carefully washed, then pitted and 2 L of water per kilogram of pulp added, and the solution heated at 80°C for 2 h. The extract obtained was then centrifuged for 30min at 5000 r.p.m to remove any cellulosic material remaining. The supernatant was then used as a carbon and energy source for lactic acid fermentation, after proper dilution [25].

II.3. Cultures

Stock cultures of *L. helveticus* were reactivated on MRS agar medium incubated for 24 h at 30°C. Two successive pre-cultures on liquid MRS medium incubated for 24 h at 30°C were carried out, and then culture Erlenmeyer flasks were inoculated with the final pre-culture at 10% inoculation level (v/v). Batch cultures were carried out in 250 mL Erlenmeyer flasks. The working volume was 100 mL and 10 g of shell was added (10% w/v). The agitation speed was 200 r.p.m, the initial pH was in the range 8–9 and the temperature was maintained constant at specified values in the range 30–42°C. The inoculation level was 10% (v/v). Culture media and all other materials used for this study were sterilized (20 min at 121°C). Two culture media were chosen, the first containing glucose and the second consisting of date's juice.

II.4. Fermentation conditions

In the first series and with each of two concentrations 80 and 300 g/L of glucose, different incubation temperatures (30, 35 and 42°C) were studied to determine the effect of increasing temperature on the efficiency of chitin recovery. In the second series, two ranges of concentrations of

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reducing sugars at different incubation temperatures were selected: 40-250 g/L at 30°C and 10-100 g/L at 35°C. For each case, the rate of demineralization (DM) and deproteinization (DP) were calculated. The comparison of the results allowed the optimization of the concentration of carbon source and fermentation temperature for maximum DM and DP, and consequently recovery of pure chitin.

II.5. Analytical methods

Samples were harvested at regular time intervals, centrifuged and the supernatants recovered for analysis. The following physico-chemical parameters were investigated: pH, glucose and Total Titratable Acidity (TTA) expresses the number of grams of lactic acid presents in one liter of sample. Its determination is based on the principle of titration with sodium hydroxide in the presence of phenolphthalein as indicator solution [27].

The solid fraction recovered at the end of culture was washed and then ambient air-dried for several days in order to determine protein and ash contents. Glucose was determined by the dinitrosalicylic acid method at a wavelength of 575 nm. Total solubilized proteins determination was carried out by the Biuret method (reading at a wavelength of 540 nm). Ashes were determined by incineration of a 1 g sample in a muffle furnace at 900°C for 2.5 h [28]. The calculation of ashes allowed the determination of the final yield of shell demineralization [29].

III. *Results and discussion*

III.1. Study of demineralization and deproteinization of shrimp shells using glucose as carbon and energy sources

To ameliorate rates of demineralization and deproteinization of shrimp shells obtained in previous work [26], many fermentation tests were performed. At each of two concentrations (low concentration: 80 g/L and high concentration: 300 g/L), different incubation temperatures: 30°C and 35°C with 80g/L of glucose; 35°C and 42°C with 300 g/L of glucose, were studied. For each case, these rates were calculated and compared.

The 1st step was carried out to study the influence of temperature (30°C and 35°C), with a low initial glucose concentration (80 g/L), on the rate of deproteinization. Figure 1 shows that pH 6-6.5 was achieved and stabilized within 24h of culture and then rises rapidly to neutral pH between 6.5 at 30°C and 7 at 35°C, corresponding to 70% and 9% of deproteinization, respectively. These tests showed clearly that the temperature of 30°C is optimal for shell deproteinization. These results were explained by the medium pH which is optimal (6-6.5) for proteases activity [30] and indicated a remarkable influence of the temperature for chitin recovery by lactic acid fermentation.

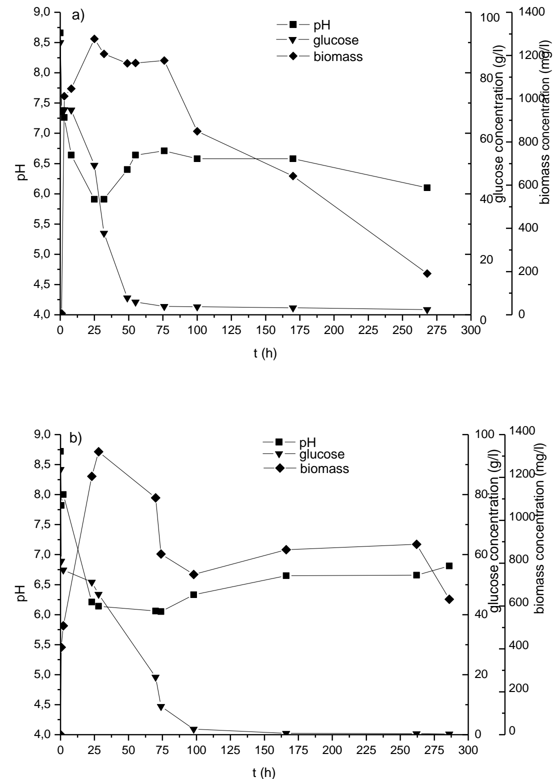

Figure 1. Effect of temperature (30°C (a) and 35°C (b)) on pH, glucose and biomass concentrations (80 g/L of glucose, fermentation volume 100 mL, 10 g shell, 10% of inoculum, agitation 200 r.p.m).

The low initial content of glucose (80 g/L), compared to 300 g/L, is responsible for the low acidification occurred in the medium (pH minimum is only 6 (Fig.1)). It is therefore evident that the rates of DM were not significant in our experiments. In addition, with 80 g/l glucose, it is noted that the kinetics of substrate consumption accompanied by acidification of culture medium by lactic acid production and cell growth are different at both temperatures (30 and 35°C). At 35°C, the time required for acidification is double than obtained at 30°C. This result indicates that acidification by conversion of glucose is favored by high temperature. It is observed that, there is a rise of pH which coincides with glucose depletion (initially 80 g/l), which explains appearance of the decline phase. This rapid rise of pH is probably due to the oxidative metabolism of bacteria [31] fostered in a medium with low glucose concentration.

Our results are in agreement with those obtained in many works. Biological treatment of minced

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scampi (*Nephrops norvegicus*) waste supplemented with glucose, using *Lactobacillus paracasei* strain A3 and after 3 days batch culture at 30°C lead to solubilization of 77.5 and 61% of protein and calcium, respectively, initially present in the waste material [32]. The fermentation of shrimp waste by *Lactobcillus plantarum* 541 with and without pH control was examined by reference [29]. Among four acids tested (glacial acetic, citric, hydrochloric and lactic acids) to control pH at the start and during fermentation, acetic and citric acids proved to be the most effective. In presence of an additional carbon source, glucose, 75% deproteinization and 86% demineralization of biowaste were achieved at pH controlled at 6.0 with acetic acid, while without pH control 68.1 and 64.1% deproteinization and demineralization, respectively were achieved. Reference [26] was obtained without pH control 60% and 76% of demineralization and deproteinization, respectively, using *Lactobacillus helveticus* to extract chitin from waste of *Parapenaeus longirostris*.

The 2nd step was carried out to ameliorate the rate of demineralization with high glucose concentration (300 g/L) at 35°C and 42°C.

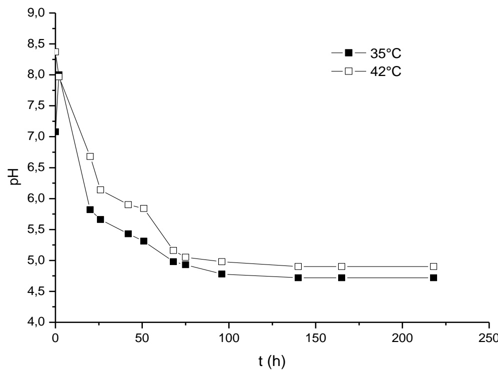

Figure 2. *Effect of temperature (35 and 42°C) on medium pH (300 g/L glucose concentration, fermentation volume 100 mL, 10 g of shell, 10% of inoculum, agitation 200 r.p.m)*

It appears from the figure 2 that pH dropped rapidly early then slowly to achieve a stable value after 100h at 35°C, pH 4.7 was obtained corresponding to 60.8% of demineralization but at 42°C, pH was 5 led to 26.1%. These results were explained by the amount of lactic acid measured (Fig. 3) and produced by *Lactobacillus helveticus.*

Low deproteinization (42.14% at 35°C and 20.22% at 42°C) was attributed to the rapid and important acidification accompanied by a high production of lactic acid (Fig. 3) in presence of high glucose concentration (300 g/L of glucose).

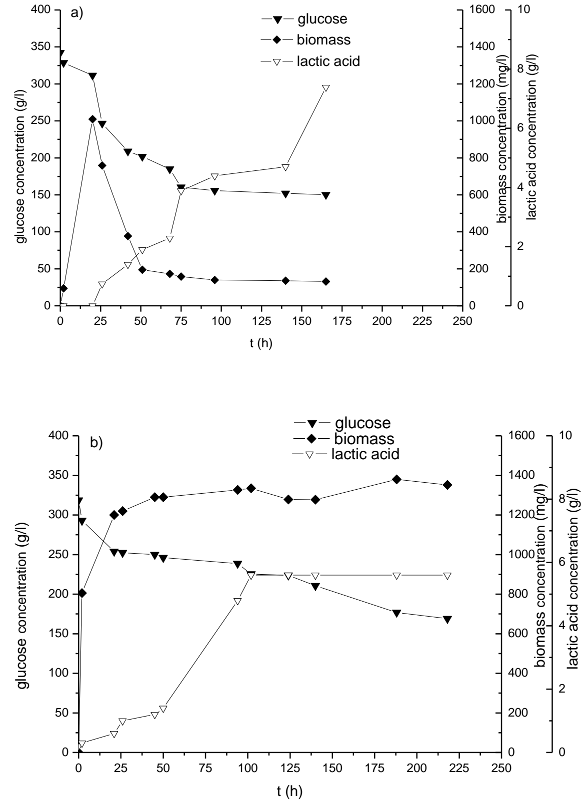

Figure 3. *Effect of temperature (35°C (a) and 42°C (b)) on glucose, biomass and lactic acid concentrations (300 g/L glucose, 100 mL fermentation volume, 10 g of shell, 10% inoculum, agitation 200 rpm)*

At both temperatures (35 and 42°C), after 24h of fermentation, it is produced a limitation of growth (Fig. 3) by accumulation of lactic acid produced by bacteria [33]. At 42°C, corresponding to the optimum temperature for growth of *Lactobacillus helveticus*, it appears the slowdown phase accompanied by a high production of lactic acid followed by stationary phase where the carbon substrate is used as a source of energy for maintaining cell which explains the cessation of production of lactic acid after 100h of culture (Fig. 3a). But at 35°C, it is revealed directly the decline phase due to high concentration of end product (lactic acid (Fig. 3)) and toxic metabolites that have accumulated in the medium [34], as calcium carbonate (Fig. 4) from shells demineralization which are, according to reference [35], toxic for microorganisms at high concentration [35].

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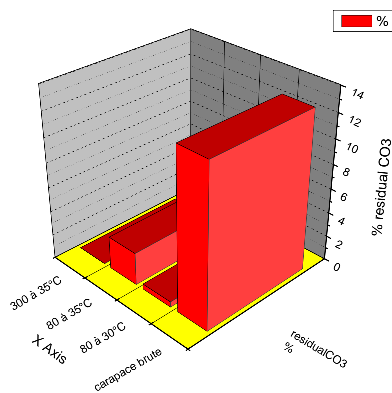
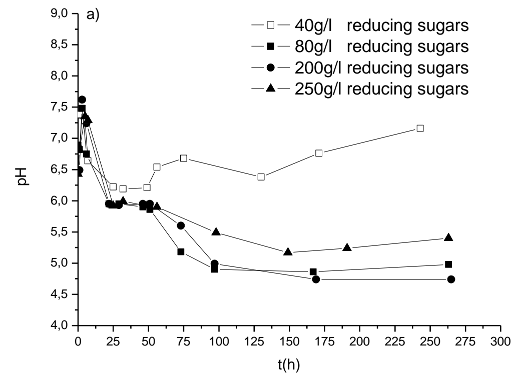


Figure 4. *Rate of calcium carbonate residual from shrimp shells fermented (100 mL fermentation volume, 10 g of shell, 10% inoculum, agitation 200 rpm)*

The calculation of DM shows that the temperature of 35°C is optimal for solubilization of minerals from shrimp shells, mainly calcium carbonate (Fig. 4). It is noticed that, there is a limit on the rate of demineralization (maximum 60%) while the end of fermentation medium contains an amount of lactic acid as shown by the results of the assay (Fig. 3). The most likely explanation is the difficulty in the solubilization of some others minerals embedded in the shell by the organic acid.
At 35°C and high glucose concentration (300 g/L) (Fig. 2), there is stability and persistence of acidity which may be explained by a fermentative metabolism due to anoxia created by high glucose concentration and accumulation of carbon dioxide by dissociation of calcium carbonate dissolved by lactic acid produced by *Lactobacillus helveticus.*

III.2. Study of demineralization and deproteinization of shrimp shells using date's juice as carbon and energy sources

On a commercial scale, glucose, starch or sucrose addition is an expensive alternative. The use of cheaper sources of carbon may be useful. In this context, we used the date's juice as fermentation medium for chitin extraction. In previous work, reference [26] using date's juice were obtained 60% of demineralization with 208 g/L of reducing sugars at temperature of 35°C and 91% of deproteinization with 80 g/L of reducing sugars at temperature of 30°C .
In this work, it has been proposed to find conditions of temperature and carbon source concentration to avoid the decoupling between demineralization and deproteinization previously observed [26]. Two ranges of concentrations of reducing sugars and different incubation temperatures were selected: high concentrations (40-250 g/L) at 30°C and low concentrations (10-100g/L) at 35°C.



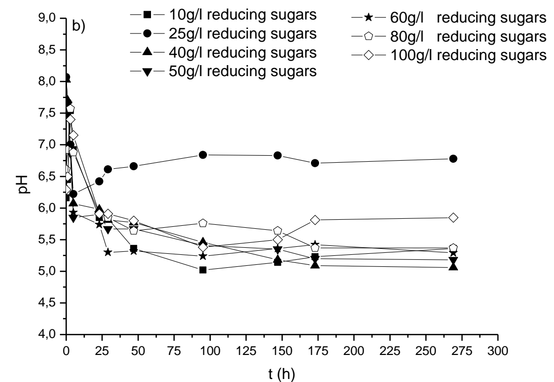


Figure 5. *Effect of reducing sugars concentrations on evolution of medium pH **(a)** (40-250 g/L) at 30°C **(b)** (10-100g/L) at 35°C (fermentation volume 100 mL, 10 g shell, 10% of inoculum, agitation 200 r.p.m).*

At 30°C (Fig. 5a), the curves of pH kinetics obtained with 80, 200 and 250 g/L of reducing sugars are completely different with that of 40 g/L of reducing sugars. In presence of glucose (300 g/L), pH decreases continuously until it reaches a minimum and stable pH (4.7), this result is in agreement with those obtained by reference [26], while with date's juice, at 30°C the pH decreases to slow down and stabilize around the value of 6 (for all concentrations of reducing sugars study), during few hours (31h) and then continues to decrease until reaching a minimum pH varying with reducing sugars concentrations (4.98, 4.74, 5.40 with 80 g/L, 200 g/L, and 250 g/L of reducing sugars, respectively). At 35°C (Fig. 5b), curves show a change in pH shape completely different from that obtained at 30°C for all levels except for curve of 10 g/L of reducing sugars: pH decreases rapidly to stabilize between 5 and 6 according to sugar contents in the medium. The rates of

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demineralization and deproteinization, calculated at the end of fermentations were shown in figure 5. The deproteinization of the shells occurs for all levels of reducing sugars from 10 g/L to 250 g/L and at two temperatures studies; it is the same for DM.

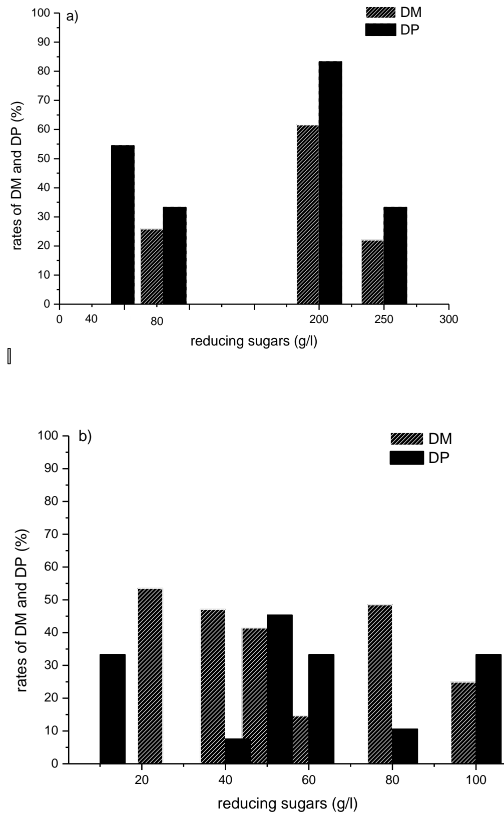

Figure 6. *Effect of reducing sugars concentrations and temperature (a) (40-250 g/L) at 30°C (b) (10-100 g/L) at 35°C on the rate of demineralization and deproteinization (fermentation volume 100 mL, 10 g shell, 10% of inoculum, agitation 200 r.p.m).*

At low reducing sugar concentration (40 g/L) and at 30°C, the demineralization is absent. This result is due to the low acidification of medium (Fig. 7b). So this decrease and stabilization of pH around 6.5 led to 55% of deproteinization. But at high concentrations, the maximum rate of DP (83%) is achieved with 200 g/l of reducing sugars at 30°C (Fig. 6a).

In other side, the demineralization begins only at 80 g/L and 25 g/l of reducing sugars at 30°C and 35°C, respectively. It should be noted that the rate of demineralization obtained with 25 g/L of reducing sugars (53%) was identical with glucose but at high concentration (300 g/L) at temperature 35°C [26]. These series of experiments show clearly that the optimum temperature for demineralization of the shell is 35°C which is in agreement with works of reference [36].

We conclude that, at temperature of 30°C, the concentration of 200 g/L reducing sugars is optimal for chitin recovery by this bioprocess. The evolution of medium pH and lactic acid concentration (Fig. 7) explains this result. The pH (Fig. 5a) has stabilized at pH around 6, during 51h, and the low amount of lactic acid (Fig. 7a) produced by the lactobacilli led to 83% DP. The large amount of lactic acid produced during the phase of decline leading to 61% DM.

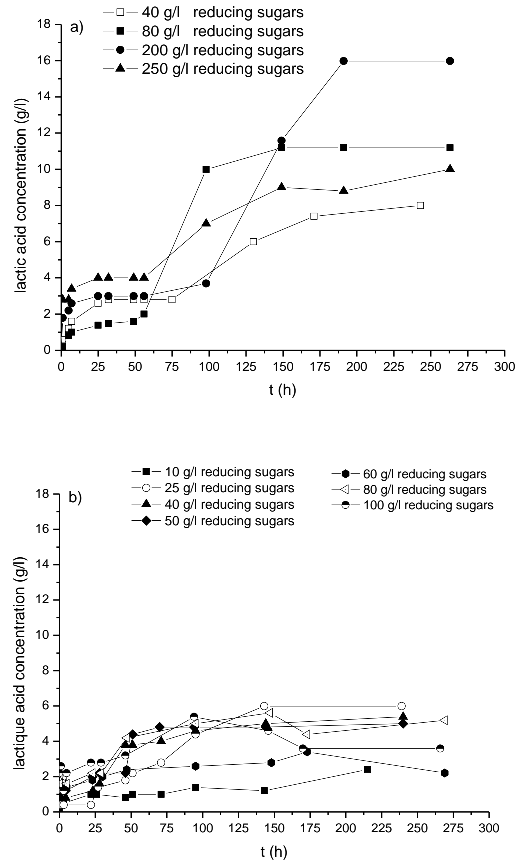

Figure 7. *Effect of reducing sugars concentrations and temperature (a) (40-250 g/L) at 30°C and (10-100 g/L) at 35°C on lactic acid concentration (fermentation volume 100 mL, 10 g shell, 10% of inoculum, agitation 200 r.p.m).*

In conclusion, the use of date juice as fermentation medium to the concentration of reducing sugars 200 g/L and incubation temperature of 30°C, allows a simultaneous deproteinization and demineralization of the shell at appreciable levels unlike the glucose-based medium and chemical treatment. This is due to the high concentration of reducing sugars in the date's juice and the presence of different mineral elements. The four major mineral constituents are Ca, P, K and Mg but it also contains traces of Fe, Al, Na, Cr, Sr, Cu, Zn, Ni, Mn and Pb. Inorganic phosphorus was previously reported to have a significant effect on lactic acid production [37]. Minerals have a positive effect on bacterial growth, playing a role in enzymes and enzymatic cofactors. This was especially the case for magnesium, one of

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the major date juice constituents [26]. In addition, calcium is known to have a positive effect on proteolytic activity of the cell [37], especially on cell-bound proteinase [39].

These results are very interesting from an economic standpoint because the waste of dates may replace glucose as it leads to better results and above in a single operation as opposed to synthetic media (DM 61% with 300 g/L glucose and 35°C and 70% DP with 80 g/L glucose and 30°C).

The representation rate of DM depending on the concentration of lactic acid product shows the existence of a relationship of proportionality between the demineralization and the acidity of the medium at 35°C: DM=-36,6+15,8×[lactic acid] (Fig. 7).

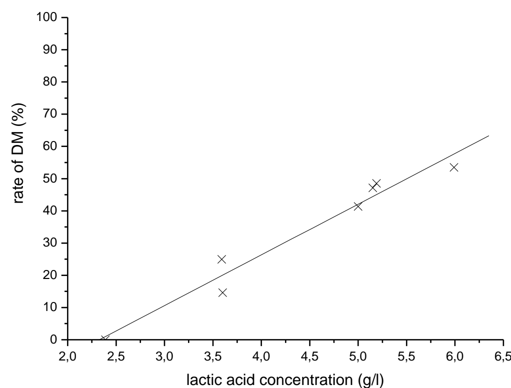

Figure 8. *Relation between the rate of DM and lactic acid concentration at 35°C (fermentation volume 100 mL, 10 g shell, 10% of inoculum, agitation 200 r.p.m).*

IV. Conclusion

…Lactic fermentation carried out using high glucose concentration (300 g/L in this work), has shown that the temperature of 35°C is optimal for maximum demineralization (60%) of the shell. Under these conditions (acidic environment), proteases activity was inhibited which explains the low deproteinization calculated at the end of fermentation (9%). While the use of low glucose concentration (80 g/L) and at 30°C led to a better deproteinization, due to the optimal pH (around 6) of proteases activity. Unlike glucose, the inoculation of *Lactobacillus helveticus* in date's juice rich in reducing sugars and various minerals necessary for lactic acid bacteria metabolism allows to a simultaneous demineralization and deproteinization of shrimp shell at 30°C with reducing sugars concentration of 200 g/L which given 83% and 61%, respectively. From our work we conclude that the use of date's juice can replace glucose for chitin recovery by biological way. These results may be explained by the presence of metallic ions which play role as co-factor for glycolyse enzymes and proteolytic enzymes.

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