Journal of Research in Chemistry

E-ISSN: 2709-9423
P-ISSN: 2709-9415
JRC 2021; 2(2): 64-70

www.chemistryjournal.net
Received: 24-06-2021
Accepted: 25-07-2021

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Preliminary investigation on kinetics on deprotenization of green crab shells for extraction of chitin

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Abstract
The green crab (*Carcinus maenas*) shell is the feedstock for numerous bio-products that are to be utilized in various applications. Chitin and Chitosan are the biopolymers from crab shells and its application in the design of medical devices such as drug delivery vehicle and tissue engineering construct. Deprotenization is one of the crucial steps in the extraction of chitin from green crab shell. The studies on the kinetic of deproteinization will be used in the reactor scale up and extraction scale up. The kinetic study clearly revealed that three step mechanism with evaluated rate constants from experimental data. The effects of temperature on the rate constant are also investigated. This investigation could lead to the scaling up the process for deprotenization.

Keywords: Chemical based extraction of chitosan, green crab shells, de-proteinization and temperature

Introduction
Carcinus maenas (L.), Green crab, is an invasive and globally dispersed species present on both the east and west coasts of Atlantic Ocean of North America, with severe negative ecological impacts on native species. Green crabs represent a plentiful, easily-harvested, and underutilized nutrient-rich biomass, although their biomass is largely unquantified and unutilized as their use in human diets is very limited. A fishery for *C. maenas* as a delicacy for humans and as a scent for seafood-based products occurs in Portugal, where the majority of the crabs is exported live to Spain for consumption or re-exports. The green crab has flourished in North America because it is able to tolerate a wide range of sea water temperatures and salinities and live in many types of marine habitats. This species of crab is a voracious predator that feeds on a variety of prey in- cluding soft shell clams, quahogs, mussels, and oysters. In addition, the green crab competes with other crustaceans for nutrient resources and habitat, and damages the ecosystem by digging in sediment around grass beds and disturbing the root systems [1].
Green Crab shells are to be utilized as a potential raw material for the production of chitin and chitosan as a biomaterial for development of medical devices and scaffolds. Chitin is the most important carbohydrate based polymer in the field of natural polymers [2]. The de-acetylated chitin called as Chitosan is soluble in acidic aqueous media and then is used in many formulations and applications such as food, cosmetics, biomedical and pharmaceutical applications. Chitosan is a biodegradable, biocompatible and non-toxic alternative to synthetic polymers that you would find in a whole bunch of different industries. The natural compound chitin/chitosan could be used as an alternative for synthetic polymers in various developments of bio products such as cosmetics, toiletries and pharmaceuticals [3].
The chitosan is produced by chemical based extraction from crab shells which has various sequence steps namely demineralization, de-protenization, deacetylation and de-coloration/de-pigmentation. Deprotenization is the crucial step in extraction process. Because it remains obscure about binding of proteins with chitin in the crab shells. As a consequence, the deproteinization is a complex process and lack of information of about interaction between protein and chitin and its chemistry in the literature. Deprotenization by alkali method such as sodium hydroxide is a common method for removal of proteins from the shrimp shells. Based on the literature review, Sodium hydroxide is chosen as a deprotenization agent for this process. This report explains the optimized concentration of sodium hydroxide, contact time and temperature of the de-proteinization process of crab shells and its limitations such process problems [4, 5].

Fig 1: European Green Crab from Sea of Atlantic Canada – Halifax, NS, Canada.

These values are taken from (1) Beth A. Fulton *et al*. 2013 "Nutritional Analysis of Whole Green Crab, *Carcinus maenas*, for Application as a Forage Fish Replacement in Agrifeeds", Sustainable Agriculture Research.

Table 1: Proximate Analysis of Green Crab Shells

Proximate Component	Green Crab mince (%) Wet Basis
Moisture	67.96±0.46
Ash	16.55±0.29
Protein	12.27±0.25
Fiber	02.87±0.15
Fat	00.21±0.07

In this table, the calcium content in the green crab shell is around 16.55% and in the de-calcification process, it should be neutralized with hydrochloric acid/ any other mineral acid. The protein content is 12.3% and it is removed by deprotenization process. The above values can vary with source and maturity of green crab and its thickness of shell [6].

Table 2: Chemical composition of Green Crab Shell

Chemical Contents	(%)
Ash	38.00
Lipids	3.23
Nitrogen	5.24
Protein	14.08
Chitin	43.9

Most of the chemical based extraction processes for chitosan from grabs shells are involved with harsh chemicals with high concentration and temperature. For example, 2M concentration of Hydrochloric acid used for demineralization process and 2M sodium hydroxide solution for de-protenization at 100 °C and exposed to 24 hrs. and these prolonged exposure of chitin and chitosan with harsh chemicals affect the quality of the polymer in terms of molecular weight and degree of acetylation. Therefore, The eco-friendly and diluted concentration of Hydrochloric acid HCl and NaOH chemical based extraction is developed for the isolation of chitosan from green crab shells.

The procedure for the production of chitin from shells of crabs includes demineralization and de-protenization process and then chitosan is produced by deacetylation of chitin. Based structure of crab shell, the calcite/calcium carbonate should be removed the crab shells to weaken the structure of crab shells. Therefore, the demineralization is carried out first step in the extraction process. Demineralization/De-calcification of crab shells is completed in 6 – 7 hrs. and treated with 0.1 M of Hydrochloric acid. It is verified by crab shells lost its brittleness. The de-mineralized Crab shells washed with deionized water to remove the residual acid content in the shell which might reduce the strength of Sodium Hydroxide solution. As a consequence, de-protenization process will be affected. De-proteinization is carried out in 1M of Sodium hydroxide solution at a temperature of 45 °C for 2 hrs.

The de-proteinized crab shell washed with deionized water and it is raw chitin material. The de-acetylation process is carried out in 50% wt. Sodium Hydroxide solution at a temperature of 95 C for 2- 3 hrs under nitrogen atmosphere. The de-acetylated crab shells are washed with hot deionized water to remove the sodium hydroxide solution. The washed de-acetylated crab shell are treated with 95% Ethyl alcohol to remove the pigments present in the shell and then washed with deionized water.

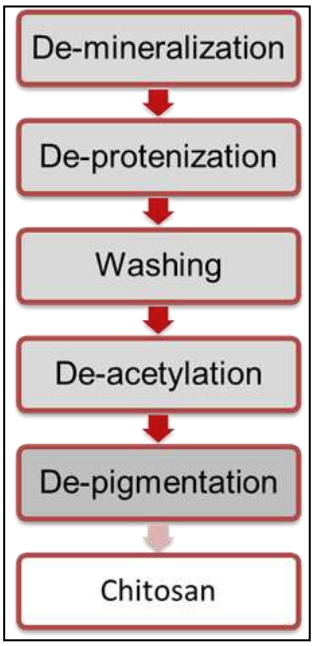

Fig 2: Sequential Steps in Extraction of Chitin/Chitosan from Green Crab Shells

This paper reveals the kinetics of the deprotinization of green crabs shells and attempted to explain the kinetics and mechanism of deprotinization of green grab shells and possible gave an explanation of influence of temperature on the kinetics and reaction mechanism.

Experimental Methods

Measurement of Protein content in the sample

Absorption Assay

The protein in the solution absorb at 280 nm due to the presence of aromatic amino acids in the proteins. Therefore, quantification of the amount of protein in a solution is possible in a simple UV-Visible spectrometer. Absorption of radiation in the near UV by proteins depends on the Tyr and Trp content (and to a very small extent on the amount of Phe and disulfide bonds). Therefore, Absorbance at 280 nm varies greatly between different proteins. Total protein

content in the sample is determined by standard curve of BSA proteins [9, 10].

Preparation of Stock Solution of BSA as standard

Bovine Serum Albumin used as standard protein and dissolved in deionized water at a concentration of 25 mg/ml for preparation of working standard solutions. The working standard is prepared at concentration of 1mg/ml, 0.8mg/ml,0.6mg, 0.4mg/ml and 0.2mg/ml. The absorbance of a protein solution is measured at 280 nm. The experiments have been performed in duplicate in order to get consistent data.

Deproteinization Process

The demineralized crab shell is washed with deionized water to remove the acidity present in the crab shell. The residual acidity in crab shell is removed by washing with water and otherwise it reacts and neutralize with sodium hydroxide solution which lowers the strength of NaOH solution. The de-mineralized shells are treated with various concentrations of sodium hydroxide solution, contact times and temperature. The 2 ml of sample from the de-protenized solution is taken every hour and measured the absorbance at 280nm.

Result and Discussion

Standard Curve of BSA

Using the above chart, the concentration of protein present in the sample has been evaluated. The standard graph is used to estimate protein released from the crab shell. In the figure 3, the amount of protein released is higher in the contact time 3 hr. and it is same for 1M and 2M concentration of NaOH. However, in this experiment, there are some limitations. The demineralized shell is treated with various concentration of sodium hydroxide solution at 45 c in beaker and kept in hot air oven set at 45 °C -50 °C. The deprotenization is a mass transfer limited reaction and therefore active mixing is required for better leaching of proteins from the shell. In addition, the prolonged treatment with sodium hydroxide solution enables removal of some pigments from the crab shell and colorizes the solution that gets chance to increase the absorbance.

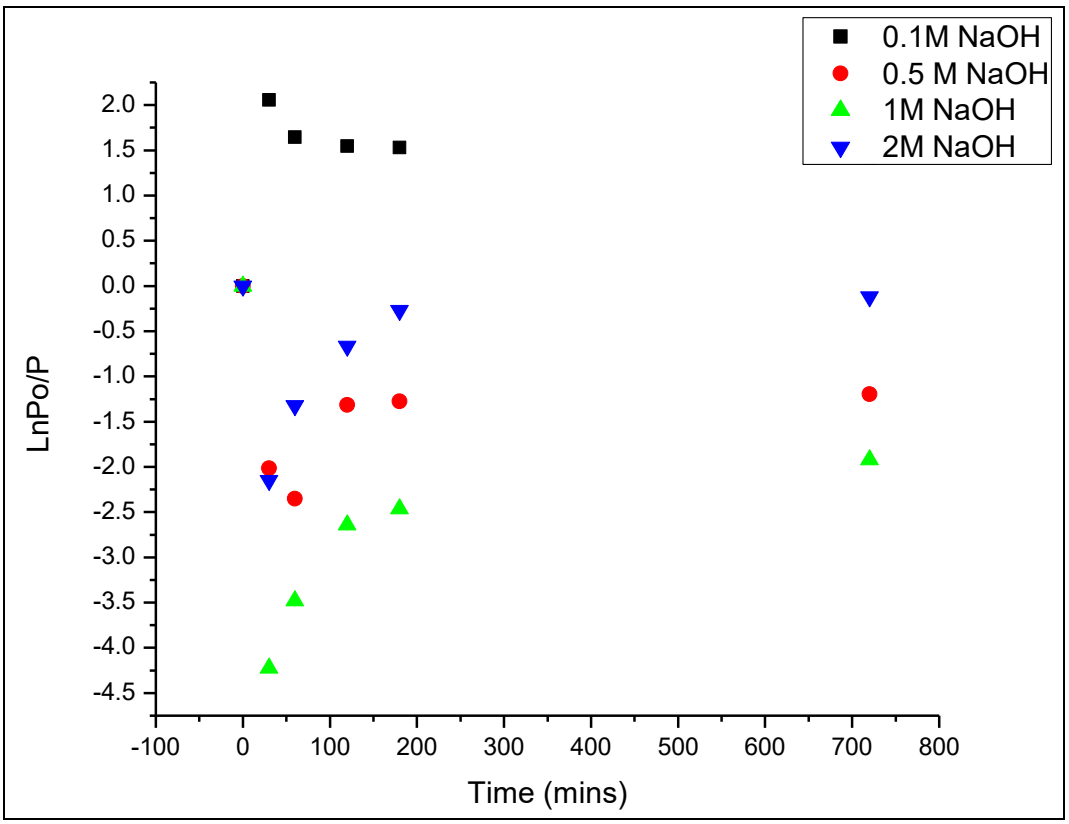

Fig 3: Plot between times versus ln (Po/P) for deproteinization of green crab shells with different concentration of NaOH.

Figure 03 reveals the increase of protein content in NaOH solution and it was increased with the NaOH concentration and time. The concentration of protein increased exponentially with time up to 200 mins and after that it reaches the equilibrium. The high concentration of NaOH enhances protein release from the crab shell.

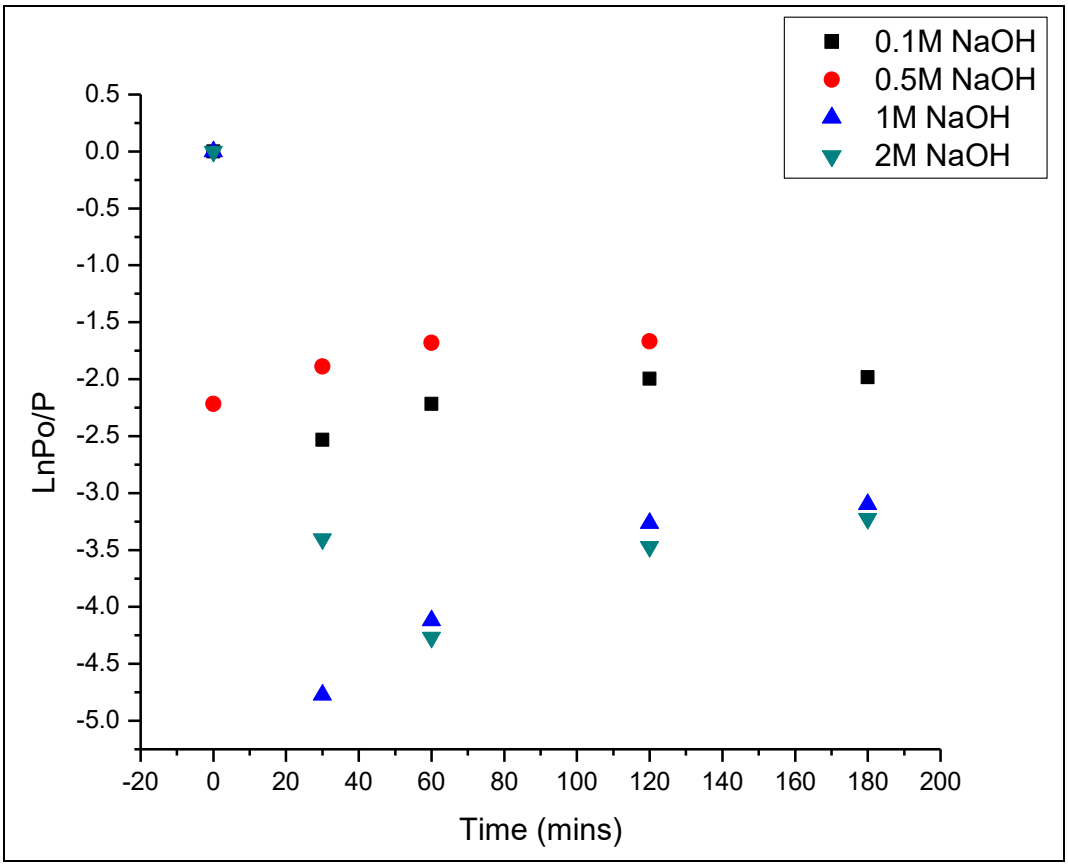

Fig 4: Plot between times versus ln(Po/P) for deproteinization of green crab shells with different concentration of NaOH.

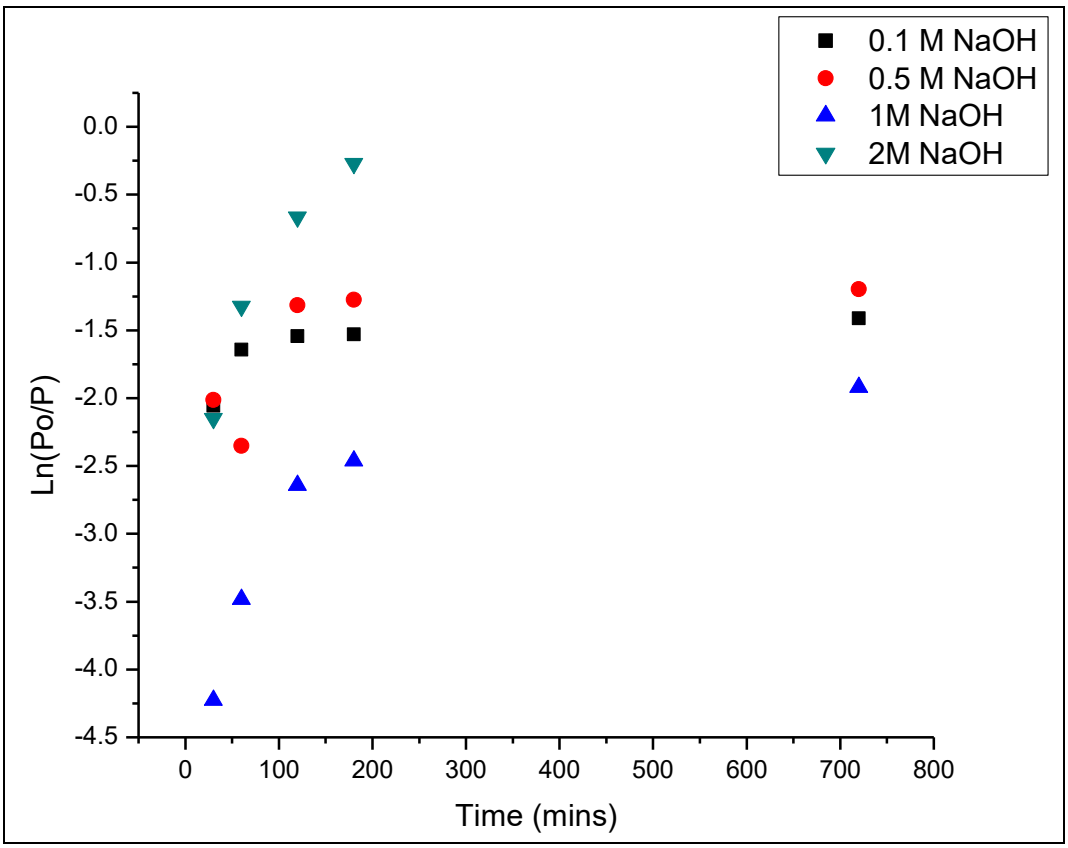

Fig 5: Plot between times versus ln(Po/P) for deproteinization of green crab shells with different concentration of NaOH.

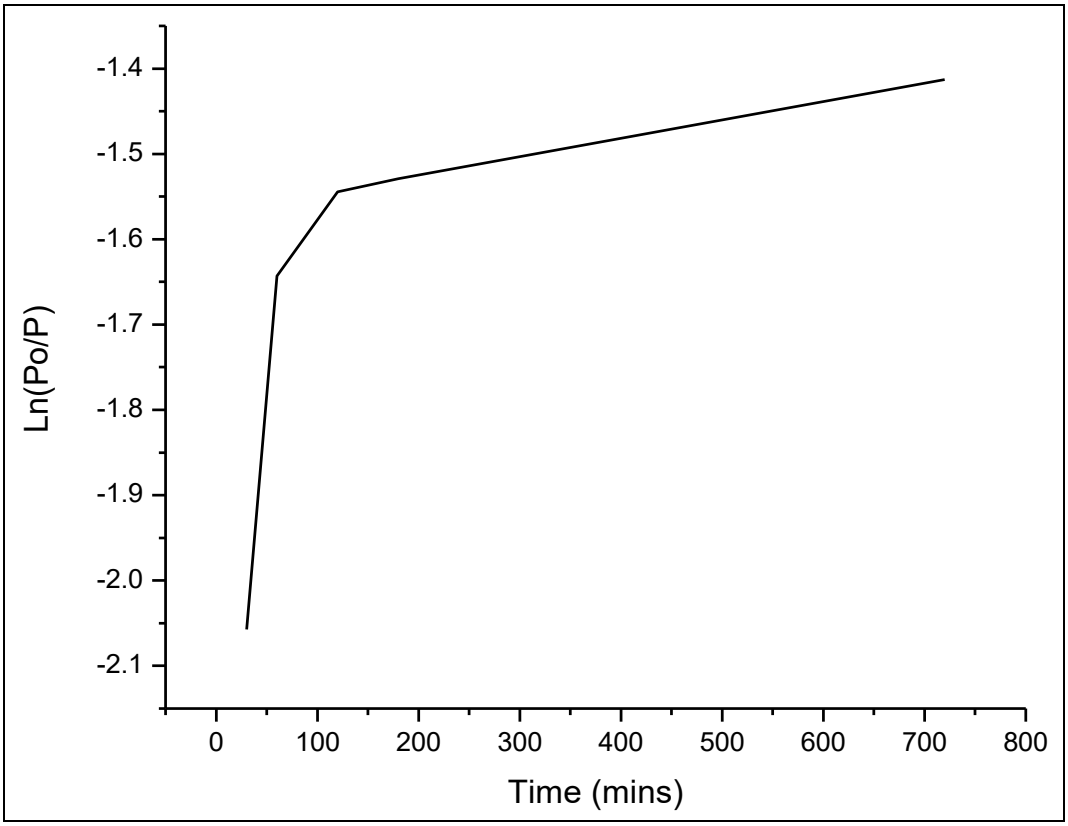

Fig 6: Plot on kinetics on deproteinization (0.1 M NaOH Treatment)

Figure 06 and 07 reveals the three stages in the reaction kinetics of deprotenization with NaOH solution. The rate constants for three stages in this kinetics have been calculated. The plot confirms that the logarithmic variation of the percentage of the proteins released from crab shell against with contact time of NaOH. It seems that the plot follows the first order kinetics and divided into three stages. These stages are shown in the plots figure 06 and 07 confirming that the protein released from the crab shell is very rapid from 35 to 100 minutes and exponential in second stage and reached equilibrium in third stage.

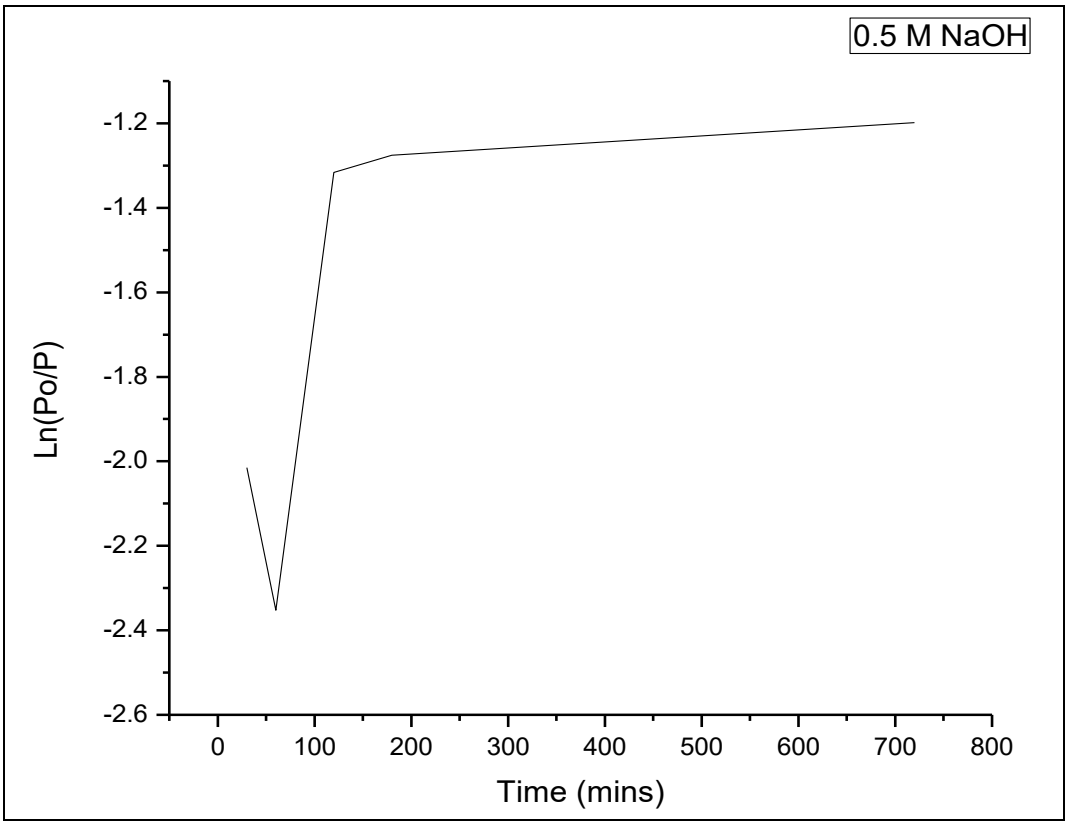

Fig 7: Plot on kinetics on deproteinization (0.5 M NaOH Treatment)

Chemical Deprotenization of Green Crab Shell

NaOH Solution is used to leach/extract the protein from the green crab shell. The mathematical model for kinetics on deprotenization is given by

$$\frac{dp}{dt} = -k\,P$$

P represents the fraction of protein present in the green crab shell before the extraction of protein with NaOH solution. K defines the rate constant of the reaction between green crab shell and sodium hydroxide solution. In this process, NaOH acts as an alkali catalyst for deprotenization. The rate constant is shown below correlation,

$$K = Bke^{-\frac{E}{RT}}$$

The protein was linked with chitosan via covalent bonds in the green crab shell and it can be broken by treatment with strong alkali such as NaOH and KOH.

Prediction of "K"

Table 3: Rate constants for deproteization of crab shells

Temperature	K_1	K_2	K_3
45 ℃	-0.025	-0.009	-0.013
65 ℃	-0.06472	-0.02151	-0.00251
85 ℃	-0.02542	-0.00612	-0.00033

Table 3 summarize the rate constants for deprotinization of green crab shell. From the table 1, it can be conclude that the variation in rate constants seems three stages in the kinetics of deprotenization. The rate of deprotinization of green crab shell with NaOH solution depends on the structure of the crab shell, the protein linkage with chitonous matrix (on the surface of the shell and interior of the shell), types of bonds interacting with chitin/chitosan such as hydrogen bonding, ionic interactions, hydrophobic interactions or covalent bonding and the composition of protein with green crab shell [7]. The proposed kinetic model for deprotinization of green crab shell is given by the following correlations.

$$\frac{dp}{dt} = -0.025Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.009\,Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.0013Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.06472Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.02151\,Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.00251Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.02542Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.00612\,Be^{-\frac{E}{RT}}\,P$$

$$\frac{dp}{dt} = -0.00033Be^{-\frac{E}{RT}}\,P$$

Effect of Temperature

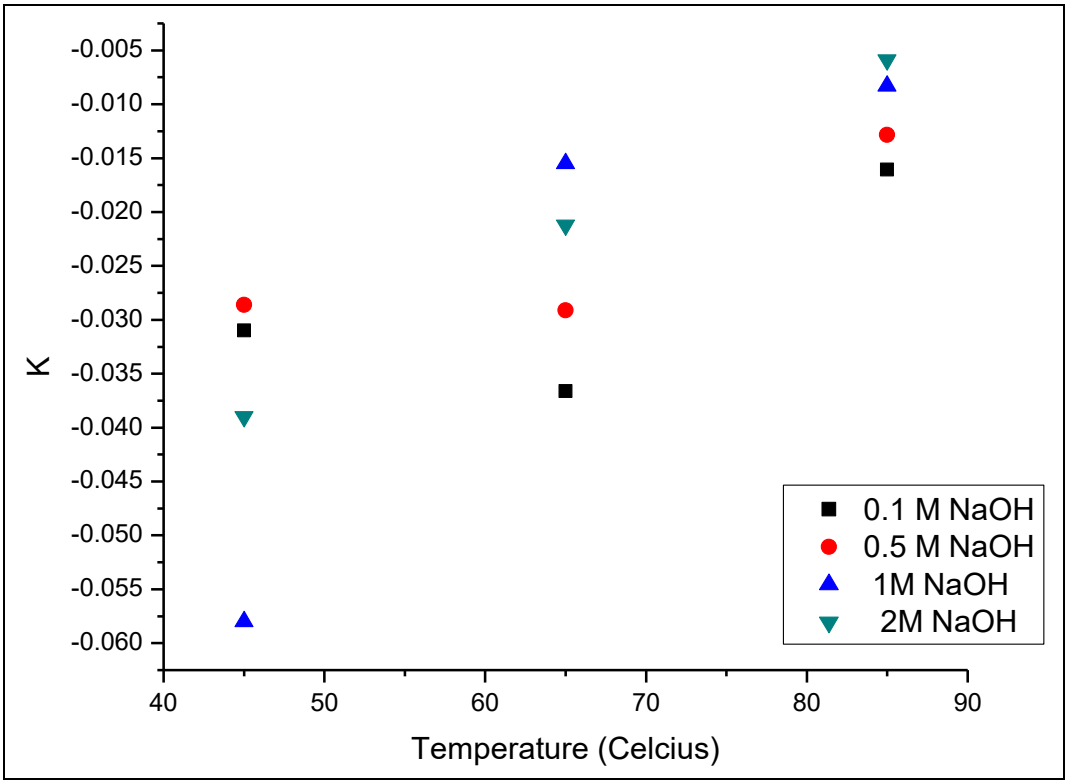

Fig 8: Plot between Rate constant and reaction temperature

Figure 08 reveals the plot between rate constant and reaction temperature confirming that there is a rise in rate constant when temperature increased and NaOH concentration increased.

Conclusion

In current investigation, the kinetics of deprotinization for green crab shell has been developed and experimental analysed. There are three stages in the kinetics of deprotinization of green crab shells. The clear mechanism on deprotenizaiton remains obscure and however, increases in temperature and NaOH concentration increase the protein released from the crab shell.

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