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Kinetics of catalyzed hydrolysis of 4-methylumbelliferyl caprylate (MUCAP) salmonella reagent

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ABSTRACT

The kinetics of chemical hydrolysis including neutral, acid- and base-catalyzed hydrolysis of 4-methylumbelliferyl caprylate (MUCAP) salmonella reagent were studied at different temperatures. The rate constants and activation parameters were determined by following the build-up of fluorescence peak of the hydrolysis product 4-methylumbelliferone (4-MU). The time scale of esterase enzyme hydrolysis caused by salmonella was compared with chemical hydrolysis as a background process.

1. Introduction

The general chemical structure of coumarins consists of a benzene moiety fused to α-pyrone rings and most of them have a very efficient fluorescing ability. Coumarin derivatives possess a wide range of applications as anticoagulants [1], antitumor [2], photosensitizers [3], anti-HIV [4], antimicrobial [5], chemosensor [6] and anti inflammatory agents [7]. Coumarin derivatives have been linked to other molecules in gene expression studies [8] as well as in salmonella detection [9]. Coumarin derivatives are also currently used as fluorogenic dyes in proteomics [10].

The photophysical properties of these compounds depend on the nature and position of a substituent group in the parent molecule and also on the surrounding media. The effect of solvents, substituents and temperature on the various photophysical properties of coumarin compounds have been reported [11–15].

On the other hand, the complicated pH-dependent fluorescence behavior of 7-hydroxycoumarin and its derivatives has attracted considerable attention from spectroscopic point of view, and efforts have been made to unravel the structures of the excited singlet-state species that are responsible for the multiple fluorescence [16–27].

There has been interest in the use of esterase activity to confirm the presence of salmonella. The majority of salmonella strains of all species and serotypes were capable of cleaving esters of the coumarin derivative 4-MU with esterase with particular activity on caprilate derivatives. Identifying salmonella in the clinical laboratories involves the use of 4-methylumbelliferyl caprylate (MUCAP); a substrate that fluoresces upon hydrolysis of the ester bond [28]. The enzyme acts on a substrate formed by an ester of 8 atoms of carbon conjugated with 4-MU, librating this last compound, which is strongly fluorescent and can be observed after 3–5 min under a UV lamp (wavelength, 366 nm). The development of a blue fluorescence over or around the colony was considered a positive result [9,29–35].

Chemical hydrolysis of MUCAP is expected to be a consecutive process that occurs alongside with enzyme-catalyzed hydrolysis. No data is available about the acid-catalyzed, base-catalyzed and neutral hydrolysis of MUCAP as a background of enzyme hydrolysis counterpart. In this communication, we report the kinetics and activation parameters of acid-catalyzed, base-catalyzed and neutral hydrolysis of MUCAP to assess the possibility of interference with enzyme-catalyzed hydrolysis caused by salmonella. We also present recorded fluorescence runs to compare MUCAP action on sterilized and inoculated agar and food samples to compare the time scales of enzyme and chemical MUCAP hydrolysis.

2. Experimental

2.1. Materials and instruments

4-Methylumbelliferyl caprylate (MUCAP test, 8 mL in heptane) was purchased from Biolife Italiana S.r.l.

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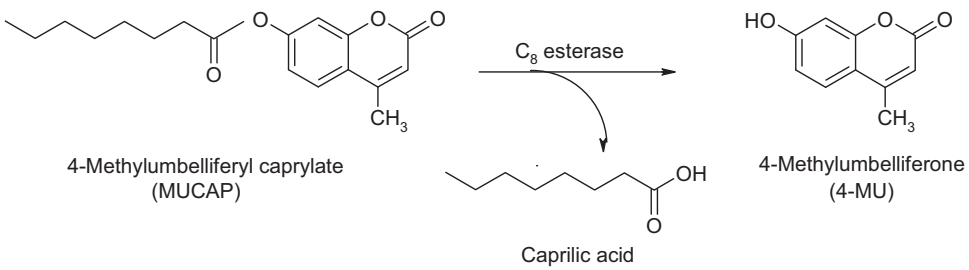


Scheme 1. Enzyme-assisted hydrolysis of MUCAP reagent yielding fluorescent 4-MU.

Steady-state emission spectra were measured using a Shimadzu RF 510 spectrofluorophotometer connected to a Haake Ultrathermostat (Julabo F10) of temperature precision ±0.1 °C using a rectangular quartz cell of dimensions 0.2 cm × 1 cm to minimize the reabsorption. UV–visible absorption spectra were measured in 1 cm quartz cells using a Shimadzu UV-160A spectrophotometer.

2.2. Kinetic measurements

2.2.1. Neutral hydrolysis of MUCAP

A test solution (0.05 mL) of MUCAP was added to a flask containing methanol (5 mL) and made up to the mark with water in 10 mL volumetric flask. The effect of temperature on neutral hydrolysis of MUCAP was studied at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C.

2.2.2. Acid hydrolysis of MUCAP

A test solution (0.05 mL) of MUCAP was added to a flask containing methanol (8 mL) and 1 mL of 10 M HCl then made up to the mark with water in 10 mL volumetric flask. The effect of temperature on acid hydrolysis of MUCAP was studied at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C.

2.2.3. Base hydrolysis of MUCAP

A test solution (0.05 mL) of MUCAP was added to a flask containing methanol (8 mL) and 1 mL of 5×10^{-4} M NaOH then made up to the mark with water in 10 mL volumetric flask. The effect of temperature on base hydrolysis of MUCAP was studied at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C.

2.2.4. Enzyme hydrolysis of MUCAP

Pre-enrichment was achieved using lactose broth, incubated for 4 h then selective enrichment was carried out using selective broth (selenite cystine broth) incubated for 18 h at 37 °C. Subcultures were made on nutrient agar and the colonies on agar were tested directly by MUCAP reagent.

2.3. Preparation of lactose broth

13 g of lactose broth were suspended in 1000 mL of cold distilled water, heated to dissolve then distributed into fermentation tubes and sterilized at 121 °C for 15 min.

2.4. Preparation of selenite broth

23 g of selenite broth were suspended in 1000 mL of cold distilled water, heated to boiling and distributed into sterile tubes. Samples were not overheated or sterilized. The medium was used the day it is prepared.

2.4.1. Pre-enrichment culture procedures

10% (wt/v) ratio of sample to sterile lactose broth was kept in sterile tubes. The pre-enrichment cultures were incubated for 4 h at 37 °C.

2.4.2. Selective enrichment

Sterile tubes containing 9 mL of freshly prepared selenite cystine broth were prepared. 1 mL volumes from the upper third of pre-enrichment broth cultures were used to minimize the product carry-over. After shaking to ensure the distribution of inoculums, the culture was incubated at 37 °C for 20 h.

2.5. Culture method

Dried plates of nutrient agar were inoculated by streaking one loop of the selective enrichment culture and incubated for 24 h at 37 °C.

2.5.1. Fluorescence measurements

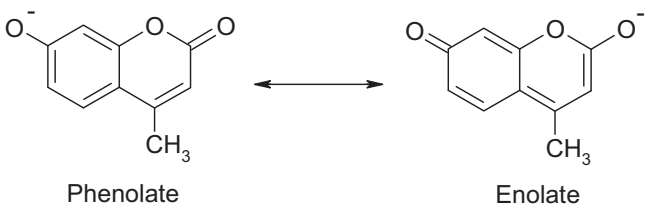
About 1 cm^2 of dried agar containing the colonies was cut and put in a fused silica cuvette at a height of 1 cm from the bottom. The emission spectrum was obtained using the 366 nm excitation light.

The judgment of positive or negative results was based on the salmonella showing continuous build up of emission. The emission was recorded immediately after adding 10 μL of MUCAP reagent. Runs were repeatedly recorded at about 3 min intervals.

3. Results and discussion

Internal conversion (ic) is a radiationless photophysical deactivation pathway of electronically excited states. It was enhanced by molecular flexibility causing a decrease in the fluorescence quantum yields. An application on the role of molecular flexibility in decreasing fluorescence efficiency is the 4-methy1umbllifery1 caprylate (MUCAP) reagent which was used in the identification of salmonella bacteria. MUCAP reagent consists of a flexible eight carbon atom ester conjugated with 4-methylumbelliferone (Scheme 1). The non-fluorescent MUCAP substrate interacted with the salmonella C_8 esterase in a specific manner leading to the release of highly fluorescent 4-methylumbelliferone that emitted strongly at 485 nm (λ_{ex} = 366 nm). A drop of the reagent was applied on the colonies on an agar surface, and the fluorescence of the colonies monitored. Fluorescence build-up indicated a positive test.

The hydrolysis of MUCAP in different media resulted in the appearance of a new fluorescence band. This band was attributed to 4-methylumbelliferone (4-MU) which resulted from the ester hydrolysis of MUCAP. The rate of MUCAP hydrolysis was both medium- and temperature-dependent. The presence of the hydroxyl group at the 7-position led to the formation of a resonant structure making MUCAP easily hydrolyzed (Scheme 2). In neutral solutions, 4-MU molecule existed in its neutral form and



Scheme 2. Phenolate–enolate resonating structures of 4-MU.

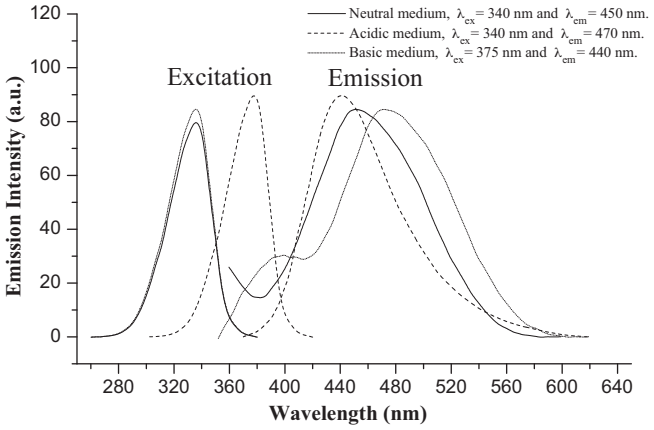


Fig. 1. Excitation and emission spectra of hydrolyzed MUCAP in different media.

absorbed close to 340 nm. Optical excitation in this case resulted in the unstable excited state shown in Scheme 3a where the proton in 7-hydroxyl group responded readily to the creation of a positive charge at the hydroxyl group and dissociated itself from the structure. The remaining excited state species may return to the ground state emitting its fluorescence (Fig. 1) [36]. In strongly acidic solutions, 4-MU absorbed at 340 nm because of the presence of the molecular cation. Excitation transferred the positive charge location from the oxygen at position-2 to the oxygen at the position-7 (Scheme 3b and Fig. 1). In alkaline solutions, the transfer of the negative charge from the 7- to the 2-positions in the excited state changed the basicity and consequently the characteristics of fluorescence (Scheme 3c and Fig. 1).

The observed first-order rate constants at different temperatures were reported in Table 1 for the reaction:

$$\mathrm{MUCAP} \rightarrow 4-\mathrm{MU} \quad (1)$$

The hydrolysis reaction follows the following general rate equation:

$$\frac{-d[\mathrm{MUCAP}]}{dt} = k[\mathrm{MUCAP}] \quad (2)$$

The integrated first-order rate law is:

$$\ln\left(\frac{[\mathrm{MUCAP}]_o}{[\mathrm{MUCAP}]_t}\right) = kt \quad (3)$$

where $[\mathrm{MUCAP}]_o$ = the initial concentration of MUCAP at time zero and $[\mathrm{MUCAP}]_t$ = the concentration of MUCAP at time t.

When $[\mathrm{MUCAP}]_o = I_\infty - I_o$, $[4\text{-MU}] = I_t - I_o$ and $[\mathrm{MUCAP}]_o$-$[4\text{-MU}] = I_\infty - I_t$

Substituting into Eq. (3) gives:

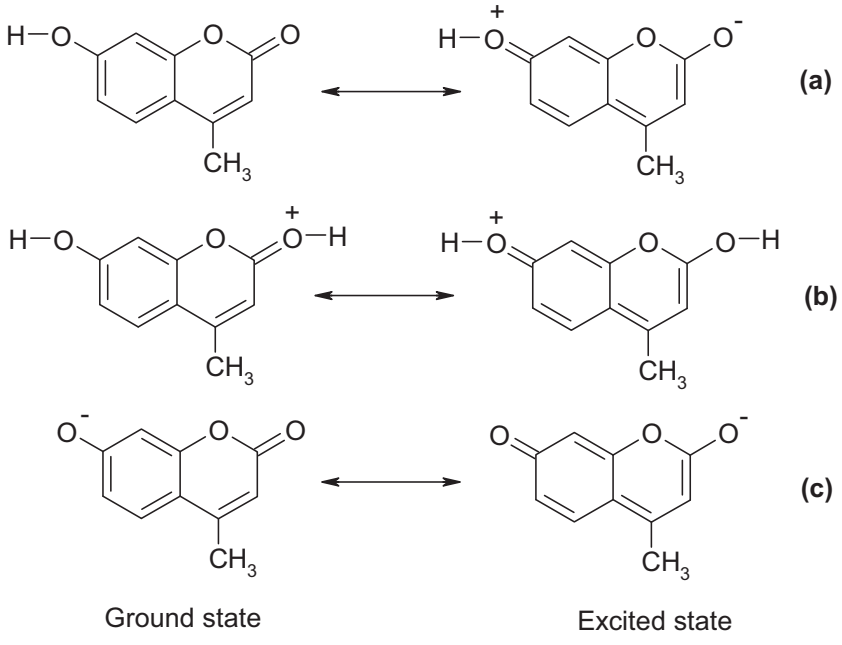
$$\ln\frac{I_\infty - I_o}{I_\infty - I_t} = kt \quad (4)$$

Therefore

$$\ln I_\infty - I_t = -kt + \ln(I_\infty - I_o) \quad (5)$$

Plotting $\ln I_\infty - I_t$ against time gives a straight line with slope of $-k$ and intercept of $\ln I_\infty - I_o$.

The kinetics of the neutral, acid and base hydrolysis of MUCAP were monitored by measuring the build-up of emission bands at 450, 470 and 440 nm, respectively versus time. Figs. 2a, 3a and 4a showed the first-order kinetic plots of $\ln(I_\infty - I_t)$ versus time for MUCAP hydrolysis. From the slopes, the corresponding rate constants were determined as shown in Table 1. Arrhenius plots yield straight lines with correlation coefficients 0.99754, 0.99736 and 0.99954 (Figs. 2b, 3b and 4b) for neutral, acid and base hydrolysis, respectively. From the plots, activation energies were calculated as 46.05, 40.08 and 46.23 kJ mol^{-1} for neutral, acid and base hydrolysis, respectively (Table 2). The enthalpies of activation ($\Delta H^{\#}$), free



Scheme 3. Charge distribution following electronic excitation of 4-MU in (a) neutral, (b) acidic and (c) alkaline media.

Table 1
The first order rate constants for MUCAP hydrolysis in different media at different temperatures.

Hydrolysis medium	k (s^{-1})				
	Temperature				
	30 °C	35 °C	40 °C	45 °C	50 °C
Neutral	1.40×10^{-5}	2.00×10^{-5}	2.53×10^{-5}	3.48×10^{-5}	4.36×10^{-5}
Acidic	2.22×10^{-4}	2.97×10^{-4}	4.07×10^{-4}	5.23×10^{-4}	6.37×10^{-4}
Basic	2.23×10^{-4}	2.98×10^{-4}	4.07×10^{-4}	5.53×10^{-4}	7.53×10^{-4}

energies of activation ($\Delta G^{\#}$) and entropies of activation ($\Delta S^{\#}$) of MUCAP hydrolysis in different media were also determined from Eyring's equation and presented in Table 3. It is interesting to note that E_a values are higher than those for diffusion-controlled reactions (10–21 kJ mol^{-1}) [37]. Therefore, they are characteristic of a chemical reaction rather than a diffusion controlled process. Further scrutiny of Table 3 reveals consistent large and negative values for entropies of activation ($\Delta S^{\#}$). This implies a highly ordered transition state (i.e. tight transition state) in the course of the hydrolysis process.

Inspection of kinetic data in Tables 1–3 shows that the rates of both acid and base hydrolysis are nearly 15 times faster than neutral hydrolysis. The enhanced base hydrolysis was due to the fact that OH^- is a better nucleophile than H_2O. In acid hydrolysis, the enhancement is due to the role of protons in making the central carbon more electrophillic and stabilizing the coumarin leaving moiety. The negative signs of entropies of activation indicate that an activated complex was obtained during the hydrolysis pathway which was more ordered than reactants. This is consistent with substitution nucleophilic bimolecular (S_N2) reaction. Since the reactions were carried out in aqueous media, the concentration of water as a nucleophile was constant and the reaction obeyed pseudo-first order kinetics.

The fluorescence spectra of agar samples before and after inoculation were measured which showed no build-up of fluorescence on agar samples that were not infected with salmonella. Fig. 5 showed fluorescence emission build-up with time for agar samples that were infected with salmonella bacteria. Comparison of the room temperature first-order kinetic rate constants of MUCAP hydrolysis in acidic medium, basic medium and

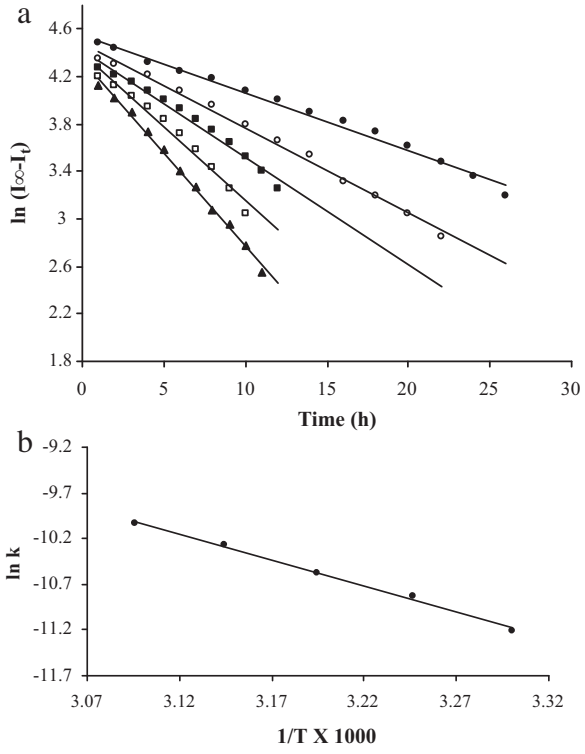


Fig. 2. (a) First order kinetic plot of MUCAP hydrolysis in neutral medium at 30 °C (●), 35 °C (○), 40 °C (■), 45 °C (□) and 50 °C (▲), λ_{ex} = 340 nm and λ_{em} = 450 nm. (b) Arrheinus plot for determination of the activation parameters for MUCAP hydrolysis in neutral medium.

Table 2
Energies of activation for MUCAP hydrolysis in different media.

Hydrolysis medium	E_a (kJ mol^{-1})	Correlation coefficient (r)	Standard deviation (SD)
Neutral	46.05	0.99754	0.03728
Acidic	40.08	0.99736	0.03375
Basic	46.23	0.99954	0.01698

Table 3
The activation parameters for MUCAP hydrolysis in different media.

Hydrolysis medium	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K)
Neutral	46.05	43.46	103.91	−193
Acidic	40.08	37.57	93.67	−185
Basic	46.23	43.72	93.55	−165

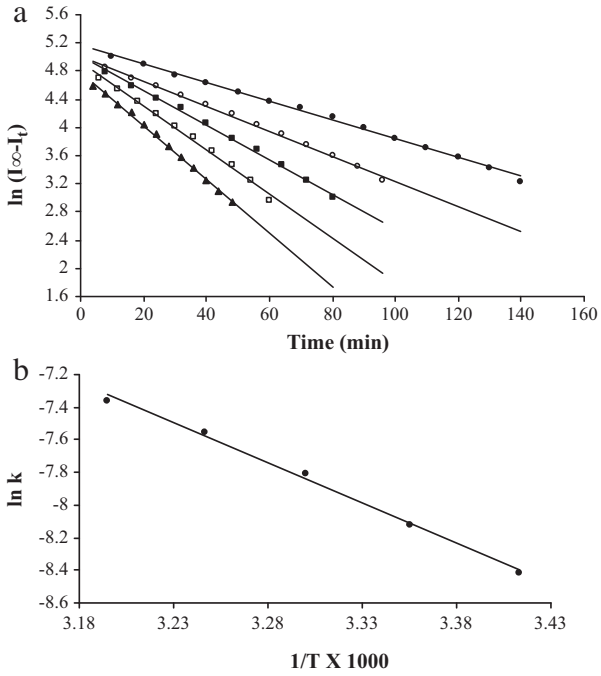


Fig. 3. (a) First order kinetic plot of MUCAP hydrolysis in acidic medium at 30 °C (●), 35 °C (○), 40 °C (■), 45 °C (□) and 50 °C (▲), λ_{ex} = 340 nm and λ_{em} = 470 nm. (b) Arrheinus plot for determination of the activation parameters for MUCAP hydrolysis in acidic medium.

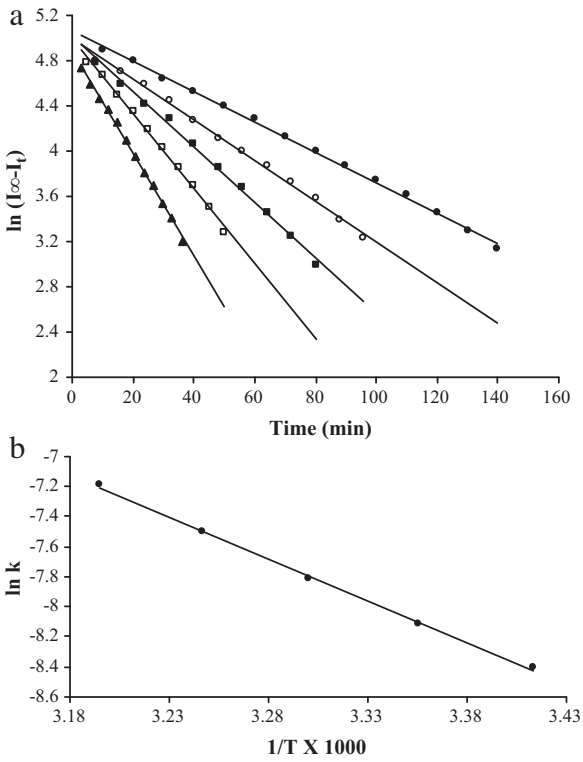


Fig. 4. (a) First order kinetic plot of MUCAP hydrolysis in basic medium at 30 °C (●), 35 °C (○), 40 °C (■), 45 °C (□) and 50 °C (▲), λ_{ex} = 375 nm and λ_{em} = 440 nm. (b) Arrheinus plot for determination of the activation parameters for MUCAP hydrolysis in basic medium.

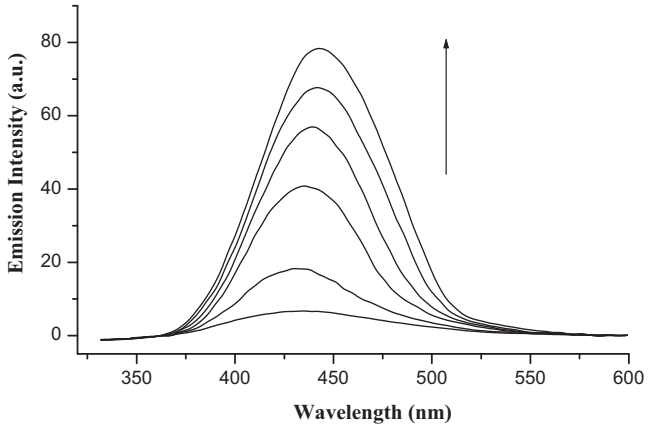


Fig. 5. Emission spectra of nutrient agar inoculated with *Salmonella typhimurium* subculture through 3 min intervals (λ_{ex} = 366 nm).

Table 4
The first order rate constants for MUCAP hydrolysis.

Hydrolysis medium	k s^{-1}(at room temperature)
Salmonella	1.48×10^{-3}
Acidic	2.22×10^{-4}
Basic	2.23×10^{-4}

salmonella environments were given in Fig. 6. The hydrolysis rate constant caused by salmonella was about 6.7 times faster than both acid and basic chemical hydrolysis rate constants (Table 4).

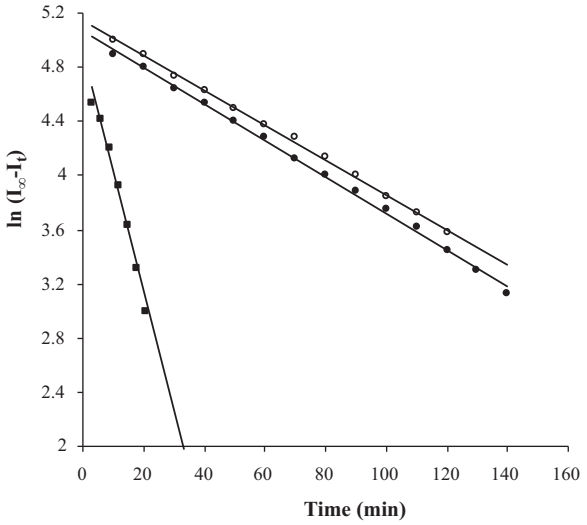


Fig. 6. First-order kinetic rate constants of MUCAP hydrolysis in acid medium (○), basic medium (●) and salmonella (■) environments.

4. Conclusions

The kinetics of the neutral, acid and base hydrolysis of MUCAP has been monitored by measuring the build-up of emission band at 450, 470 and 440 nm, respectively versus time. The rate of MUCAP hydrolysis is both medium- and temperature-dependent. The rates of both acid and base hydrolysis were nearly 15 times faster than neutral hydrolysis. The hydrolysis rate constant caused by salmonella was about 6.7 times faster than both acidic and basic chemical hydrolysis rate constants.

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