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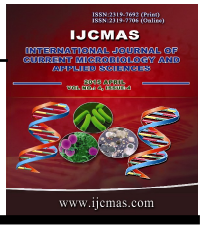

Original Research Article

Kinetic Studies of HMF Formation and Diastase Activity in Two Different Honeys of Kashmir

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K e y w o r d s

Honey, HMF, Diastase activity, V_{max}, K_m

A B S T R A C T

The main reasons of heating honey are to prevent the crystallization of honey and the inhibition of microbial growth, however the thermal treatment can have negative effects on the quality factors of honey (HMF and Diastase activity).In this particular study our objective was to study the kinetics of HMF formation and Diastase activity by applying isothermal heat treatment with different time-temperature combinations. Mustard and mixed floral honey samples were heated at 40°C,50°C,60°C,70°Cand 80°C for 5to 25minutes and HMF formation and diastase activity was determined at the above mentioned temperatures. To calculate the kinetic parameters for HMF formation Arrhenius model was used and for the calculation of V_{max} and K_m for the diastase activity Line weaver Burk method was used. The kinetic parameters for both the honey samples were found to be totally different. The HMF level increased regularly from 5 to 25 minutes for both the honey samples at each and every temperature. The HMF level increased at the speedy rate at higher temperatures like 70°C and 80°C indicating the temperature dependence on HMF formation. The diastase activity first increased from 40°C-50°C and then decreased almost regularly with the increase in temperature. It was also noticed that V_{max} and K_m values of the diastase enzyme changed with change in temperature, V_{max} first increased upto 50°C and then decreased from 60°C – 80°C indicating that diastase activity is maximal between 40°C and 50°C and beyond 50°C diastase activity reduces with the further increase in temperature, thus can be used as a quality parameter

Introduction

The Codex Alimentarius Commission (1981) defines honey as the natural sweet substance produced by honey bees from nectar of blossoms or from secretions of living parts of plants or excretions of plant sucking insects on the living part of plants, which honey bees collect, transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature. Honey is an extremely complex mixture of carbohydrates that is found naturally (Swallow and Low, 1990)

with almost 70–80% sugars, 10–20% water and other minor constituents such as organic acids, mineral salts, vitamins, proteins, phenolic compounds, lipids and free amino acids (Gomes *et al.,* 2010; Ouchemoukh *et al.,* 2007). Honey processing frequently requires heating both to reduce viscosity, and to prevent crystallization or fermentation (Singh *et al.,* 1988). However heating of honey has got some detrimental effects on the quality of honey. The detrimental affect can be measured by the measurement of quality parameters like HMF formation and diastase activity (Bodganov *et al.,* 1997).

HMF formation in honey is affected by several factors: temperature and time of heating (Bath and Singh, 1999; Piro *et al.,* 1996; White, 1978); storage conditions; use of metallic containers (Cherchi, *et al.,* 1997; Kubis and Ingr, 1998; Papoff *et al.,* 1995; Sancho *et al.,* 1992; White, *et al.,* 1964) and the chemical properties of honey, which are related to the floral source from which the honey has been extracted, these indicate pH, total acidity, mineral content (Anam and Dart, 1995; Bath and Singh, 1999; Hase *et al.,* 1973; Singh and Bath,1997, 1998). Diastases are a group of starch-digesting enzymes that include □- and β-amylase.

The enzyme □-amylase hydrolyses starch chains at random locations, producing a variety of dextrins, and β-amylase splits the reducing sugar maltose from the end of the starch chain (Crane, 1975). Very low diastase activity has been shown to indicate that the honey has been exposed to unfavourably high temperatures (Schade *et al.,* 1958).

According to the Honey Quality and International Regulatory Standards, from the International Honey Commission, the diastase activity must not be less than or equal to 8, expressed as diastase number (DN). The Codex Alimentarius (2001) and International Honey Commission (2002) set the maximum concentration of HMF to 40 mg/kg for honey from non-tropical regions and 80 mg/kg for honey from tropical regions.

Extremely high (>500 mg/kg) HMF values demonstrate adulteration with invert syrup (Coco *et al.,* 1996). However there is lack of data which correlates the effect of temperature on kinetics of the HMF formation and Diastase activity in Indian honey.

In this particular study HMF formation and diastase activity at different isothermal time temperature-combinations were determined. The kinetic parameters were also determined for each and every time temperature combinations. The HMF formation and Diastase activity kinetics (V_{max} and K_m) were correlated with temperature.

Therefore the aim of the study was to find out the effect of temperature on kinetics of HMF formation and Diastase activity.

Raw Materials

Honey samples

Two honey samples were taken from local market of Kashmir viz. mixed floral honey and mustard honey.

Descriptive analysis

Determination of HMF

Hydroxymethylfurfural was estimated by WHITE method**.** The method determines the concentration of 5-(hydroxymethyl-)furan-2-carbaldehyde. The result is usually expressed in milligrams per kilogram.

Diastase activity

Diastase activity was determined by Schade method.

Heat-treatment of honeys

Thermal kinetics of two samples of honey was studied by isothermal heating at selected temperatures (40°C, 50°C, 60°C, 70°C and 80 °C) for a residence time of 5 to 25 minutes. The samples were sealed in vials and immersed in thermostatic water bath during preset times (5, 10, 15, 20 and 25 min.) following the method described by Weemaes *et al.* (1999) and Ahmed *et al.* (2002).

The desired temperature was considered to have been achieved when the temperature of water bath reached that level. The samples were transferred to cold-water container immediately after the thermal treatment.

Kinetic calculations

Kinetic parameters like order of the reaction, K for the reaction for HMF were calculated according to Labuza and Riboh (1982) as described

$C = C_0 \exp(-k_T t)$

Where, C_0 is the total initial concentration of HMF (mg kg^{-1}), k_T is the reaction rate constant (min^{-1}), C is HMF concentration after t minute heating at a given temperature (mg kg^{-1}), and t is the isothermal heating time. Activation energies (Ea kcal mol-1) were calculated from rate coefficients at different temperatures by applying the Arrhenius equation.

Diastase activity kinetic parameters like V_{max} and K_m were calculated by applying Lineweaver-Burk method.

Other empirical models applied

Name of the model	Equation	Reference
Lewis	$C_r = \exp(-kt)$	Krokida *et al.*, 2002; Kabganian *et al.*, 2002
Page	$C_r = \exp(-kt^n)$	Midilli *et al.*, 2002 Kabganian *et al.*, 2002 Cronin and Kearny, 1998
Henderson and Pabis	$C_r = a \exp(-kt)$	Kabganian *et al.*, 2002
Logarithmic	$C_r = a \exp(-kt)+c$	Togrul and Pehlivan, 2002

Result and Discussion

HMF

HMF contents were relatively low at lower temperatures (40°C, 50°C) for up to first ten minutes (for all operations) in both honey types as shown in table 1 and 2 HMF concentrations were found lower than 40 mg kg-1 over 80°C heated for 25 minutes in both samples. HMF formation and kinetics was very different according to the origin of honey.

At the treatment temperature of 40°C the HMF level increased regularly in both the samples, mustard and mixed floral honeys from 5 to 25 minutes and at the end of heating process the HMF values increased up to 3.66 mg/kg and 4.04 mg/kg in mustard and mixed floral honeys respectively. For the first ten minutes there was a little change in HMF concentration increase, but after 15 minutes change in concentration was quantifiable in both the samples. Kinetic parameters for the both samples were

calculated according to the above mentioned formulae and were found to be different for the both samples. Mustard sample heated at 40°C followed zero order kinetics and mixed floral sample followed first order kinetics.

At 50°C Kinetic parameters were found different again for both the samples mixed floral sample heated at 50°C followed first order kinetics and mustard sample at the same temperature followed zero order kinetics with different k values.

At 60°C both the samples show higher level of HMF formation in comparison at 40°C and 50°C as shown in table. At this particular temperature the kinetic trend for both the samples change that is kinetic order changes in comparison to previous temperatures now mixed floral sample followed zero order kinetics and the mustard sample followed first order kinetics.

The HMF level increases at speedy rate as is evident from the tables 1 and 2. At the end of heating period at 80°C the HMF level for the mixed floral sample was 24.7mg/kg and 6.8mg/kg for the mustard sample indicating that with the increase in temperature beyond 70°C the HMF formation increases at the speedy rate. Mixed floral sample followed at both the temperatures at 70°C and 80°C zero order kinetics while the mustard floral sample followed 1^{st} order kinetics.

Fallico *et al.* (2004) studied HMF formation in different honeys during thermal process at 50, 70 and 100 °C up to 60 h. In terms of floral honey type, they calculated Ea values between 32.5 and 43.6 kcal mol^{-1} using pseudo-first-order rate coefficients.

Irfan Turhan *et al.*, (2008) also studied HMF formation in different honeys during isothermal heating process at75°C, 90° and 100°C. For the floral honey type the calculated E_a value of 22.32 kcal ^{mol}. Our results of E_a value were close to the previously reported values. For the mixed floral honey it was found to be 8.057$kcal^{-mol}$ and for the mustard honey it was found to be 2.3$kcal^{-mol}$.

Statistic analysis

The statistic analysis clearly showed that HMF levels in honey samples, heated at 40 °C to 80 °C, were significant correlated only with time of heating in both samples of honey. The statistical models formulated were significant for all honey samples, with the extreme values of mustard honey (R^2 of 86.1% and 99.2%). Logarithmic model and Handerson and Pubis model showed statistical significance other models were also tested but they showed no statistical significance.

Statistical analysis was most significant at temperatures of 70 °C and 80 °C for both the samples.

Diastase activity

Diastase activity, expressed as diastase number (DN) of two honeys samples at isothermal heating temperature (T) and isothermal heating time (t) (tables 3 and 4).

From the results it was clear that Diastase activity increases when the temperature was increased from 40 °C to 50 °C for both the samples although the increase in Diastase activity was not regular. The increase in Diastase activity indicates that the diastase activity is maximum between 40 °C and 50°C.

Table.1 HMF of mixed floral honey as affected by time and temperature

{HMF(mg/kg)} MIXED FLORAL HONEY							
Temp °C ↓	**Time(min)**					**R^2 1st order**	**k 1st order**
	5	**10**	**15**	**20**	**25**		
40	1.65 ± 0.04	1.68 ± 0.05	2.54 ± 0.11	4.59 ±0.54	4.04 ±0.40	0.98737	0.35116
50	2.99 ± 0.66	4.34 ± 0.30	7.06 ± 0.24	8.93 ±0.37	7.91 ±0.92	0.84382	0.05335
60	4.99 ± 0.99	9.58 ± 1.20	12.43 ±0.98	11.36±2.19	15.29±2.44	0.86005	0.44800
70	5.38 ± 2.52	8.48 ± 2.04	11.69 ±0.30	13.61±1.66	15.79±2.03	0.98551	0.51040
80	9.04 ± 1.51	15.37 ± 1.34	18.69 ±1.44	22.59±0.90	24.71±2.09	0.96699	0.77060

Table.2 HMF of mustard honey as affected by time and temperature

{HMF(mg/kg)} MUSTARD HONEY							
Temp °C ↓	**Time (min)** →					**R^2 1st order**	**k 1st order**
	5	**10**	**15**	**20**	**25**		
40	0.90 ± 0.01	0.95 ± 0.01	2.08 ± 0.09	3.95 ±0.10	3.72 ±0.08	0.88126	0.16920
50	1.02 ± 0.08	1.13 ± 0.06	2.77 ± 0.14	4.02 ±0.10	4.11 ±0.04	0.84789	0.17400
60	1.37 ± 0.02	1.44 ± 0.06	2.40 ± 0.06	4.90 ±0.18	4.60 ±0.04	0.88235	0.07557
70	1.61 ± 0.04	2.00 ± 0.09	2.68 ± 0.04	3.91 ±0.06	5.69 ±0.05	0.97436	0.07824
80	2.40 ± 0.08	2.88 ± 0.16	3.78 ± 0.13	4.62 ±0.38	6.95 ±0.15	0.97436	0.49009

Table.3 Diastase activity of mixed floral honey as affected by time and temperature

{Diastase Number (DN)} MIXED FLORAL HONEY									
Temp ↓ ℃	**Time (min) ⟶**					**Line Weaver Burk method**		**Augustinssan Method**	
	5	**10**	**15**	**20**	**25**	**V_{max} (M/s)**	**Km (M)**	**V_{max} (M/s)**	**Km (M)**
40	13.69±0.76	13.93±1.36	14.03±0.11	14.13±1.24	14.02±0.30	0.00020	-13.65103	0.00020	-13.65123
50	14.09±0.25	14.33±0.59	14.72±0.89	15.7 ± 0.40	15.26±0.35	0.00070	-13.60657	0.00071	-13.57638
60	10.2 ± 0.00	9.80 ± 1.25	8.50 ± 0.69	5.30 ± 0.82	5.60 ± 1.41	0.01800	-22.83855	0.01273	-18.20965
70	8.21 ± 0.99	7.13 ± 0.28	5.71 ± 1.41	4.92 ± 1.00	3.22 ± 1.00	0.00546	-11.11411	0.00542	-11.08109
80	6.23 ± 0.31	5.12 ± 0.86	3.16 ± 0.28	2.90 ± 0.80	2.11 ± 0.30	0.01790	-12.67651	0.01991	-15.96875

Table.4 Diastase activity of mustard honey as affected by time and temperature

{Diastase Number (DN)} MUSTARD HONEY									
Temp ↓ ℃	**Time (min) ⟶**					**Line Weaver Burk method**		**Augustinssan Method**	
	5	**10**	**15**	**20**	**25**	**V_{max} (M/s)**	**Km (M)**	**V_{max} (M/s)**	**Km (M)**
40	22.80±0.00	23.22±0.10	23.46±0.95	24.22±0.16	24.56±0.85	0.00104	-22.44965	0.00104	-22.44892
50	25.22±0.72	25.89±1.41	27.23±0.32	26.23±0.95	26.00±0.52	0.00189	-23.00405	0.00214	-22.59507
60	17.00±1.41	15.43±0.57	14.97±0.76	14.07±1.13	13.01±0.99	0.00354	-18.07776	0.00348	-18.02728
70	14.23±0.70	14.23±1.10	12.00±1.27	13.55±0.54	11.21±0.99	0.00435	-16.73860	0.00429	-16.68875
80	7.33 ± 0.42	8.02 ± 0.99	5.00 ± 0.57	3.01±1.06	3.23 ± 0.96	0.04569	-19.97033	0.04701	-20.37994

Fig.1 Zero order HMF activity at different isothermal temperatures (mixed floral honey)

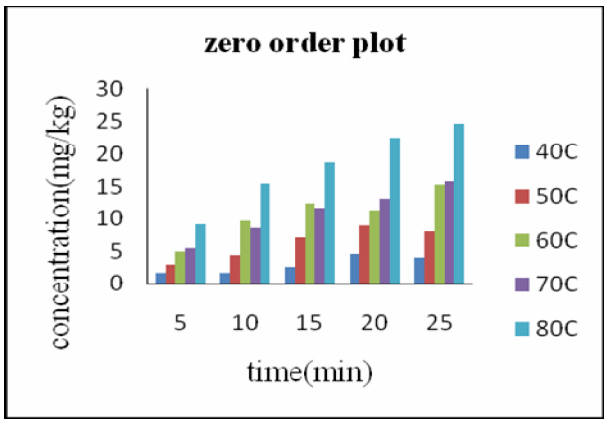


Fig.2 First order HMF activity at different isothermal temperatures (mixed floral honey)

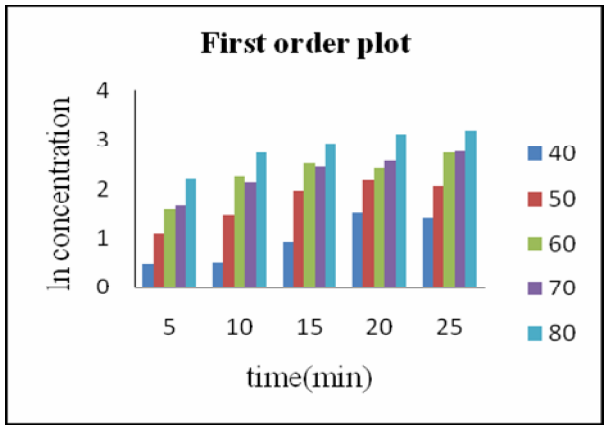


Fig.3 Zero order HMF activity at different isothermal temperatures (mustard honey)

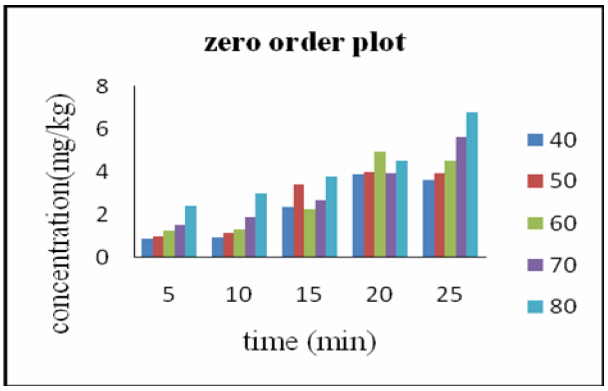


Fig.4 First order HMF activity at different isothermal temperatures (mustard honey)

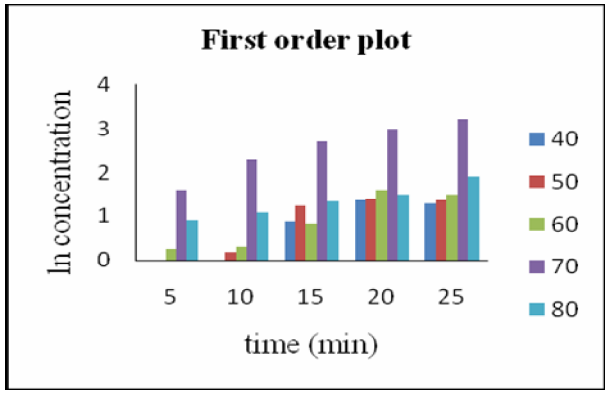


Fig.5 Diastase activity at different isothermal temperatures (mixed floral honey).

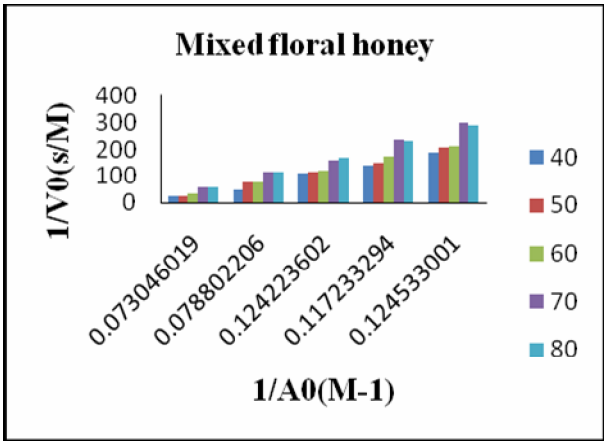
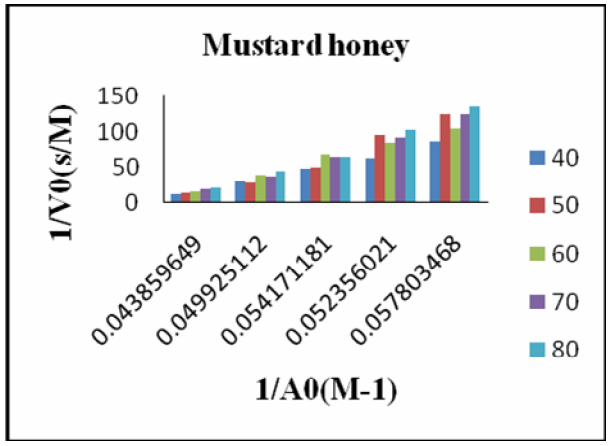


Fig.6 Diastase activity at different isothermal temperatures (mustard honey).



It was also clear from the V_{max} that diastase activity increases from 40 °C to 50 °C as V_{max} increased from 0.000201(M/s) at 40 °C to 0.000695(M/s) at 50 °C for the mixed floral honey sample. Same trend was followed by the mustard honey sample but the difference was that the increase in V_{max} was less in comparison to mixed floral honey sample. After the treatment temperature of 60 °C the diastase activity decreased continuously up to the treatment temperature of 80 °C. From the treatment temperatures (40 °C to 80 °C) the maximum and abrupt decrease in Diastase activity was at 80 °C. It was also noticed that at each and every time-temperature treatment combination near treatment times of 20 and 25 minutes the decrease in diastase activity was not regular. Such changes in diastase activity may be due to the modification of the enzymatic activity, brought about by the structural changes in enzyme molecules, promoted by heating during the isothermal heating, in samples maintained at different isothermal temperatures. As a consequence, the reaction towards complete denaturation did not occur to any large extent which results either in less Diastase activity or increasing Diastase activity at certain times. This is in accordance with the report of Kuwajima (1989) for which in the transition stage, protein maintained its native-like secondary structure, but has its tertiary structure disrupted. This variable behavior agrees with that reported by Richardson and Hyslop (1992) on the reversible inactivation of enzymes according to treatment conditions. Such is the case for alkaline phosphatase in milk and peroxidases and lipoxygenase in fruits and vegetables.

Tosi *et al.* (2008) also studied the effect of heating on diastase activity at isothermal temperatures 60 °C, 70 °C, 80 °C, 90 °C and 100 °C and reported that during isothermal heating, all samples showed a decrease of the diastase activity at short heating times. However, an activity recovery occurs in medium temperature treatments at longer times, which is in accordance with our study. No study has been found for the calculation of V_{max} and K_m values for Diastase activity during thermal processing of honey at different tempratures. However V_{max} and K_m was calculated by Shobana *et al.* (2009) for the inhibition kinetics of α-glucosidase and pancreatic amylase of finger millet revealing a non-competitive type of inhibition.

Statistic analysis

The statistic analysis clearly showed that Diastase activity in honey samples, heated at 40 °C to 80 °C, was significant correlated only with time of heating in both samples of honey. The statistical models formulated were significant for all honey samples, with the extreme values of mustard honey (R^2 of 78.1% and 98.2%). when the isothermal heating was longer, DN increase was verified (values in Table 3 and 4). Such DN increase exceeded the initial DN value (at t = 0), though it did not reach the value of the control sample. Some increased DN values were statistically significant. Logarithmic model and Handerson and Pubis model showed statistical significance other models were also tested but they showed no statistical significance.

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