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Effect of Different Storage Conditions on HMF, Diastase and Invertase Activity in *A. florea* Honey from Jalgaon District, North Maharashtra, India

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Author's contribution

The sole author designed, analysed, inteprted and prepared the manuscript.

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ABSTRACT

The present study aimed to reveal the effect of different temperatures and storage duration on the Hydroxymethylfurfural (HMF) concentration, diastase, and invertase activity of *Apis florea* honey produced from Jalgaon district, North Maharashtra, India. For the assessment of HMF, diastase and invertase activity, samples were stored at 25^0C, 35^0C, 45^0C, 55^0C and 65^0C for the duration of 1, 3 and 5 h, and the variation in these contents were determined quantitatively. According to the results, heating temperatures of 55^0C and 65^0C had adverse effects on the concentration of HMF, diastase, and invertase activity compared to honey treated at 25^0C, 35^0C, and 45^0C. On the basis of duration of heating time, it was observed that temperature increased the HMF content while decreased the diastase and invertase activity in varying proportions. The concentration of HMF increased from 33.55 (1.15%) to 48.21 (45.4%) mg/kg, when stored at 65°C for 5 hours, suggesting

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that HMF levels rise with storage temperature and time. The enzymatic activity of diastase and invertase reduced by 59.9% and 72.1%, respectively, after 5 hours of storage at temperatures of 55^0C and 65^0C, indicates deactivation of diastase and invertase enzymes at high temperature and prolong storage duration. Therefore, it was concluded that during processing and storage of honey, heat treatment at higher temperatures (55^0C and 65^0C) may affect the HMF content and enzyme activity, which reduces honey quality and lifespan. However, the storage or processing temperatures should remain upto 45^0C to preserve HMF and enzymatic integrity which keep the quality and freshness of *Apis florea* honey.

Keywords: A. florea honey; HMF; diastase and invertase activity; storage temperature; Jalgaon; North Maharashtra; India.

1. INTRODUCTION

Honey is a naturally occuring sweet syrup, widely recognized worldwide for its nutritional value and safety for human consumption (Bogdanov et al. 2008). There are more than 22 distinct sugars in honey, of which fructose and glucose are the major sugars (Bogdanov et al. 2004). Honey also contains many other substances, including pollen, amino acids, hydroxymethylfurfural (HMF), starch-digesting enzymes (invertase, diastase, and glucose oxidase), minerals, ethers, organic acids, and phenolic compounds (Sereia et al. 2017). Due to its complex chemical nature, honey exhibits antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties, fulfilling the high demand in the medical and pharmaceutical sectors (Battino et al. 2013). In addition to its medicinal value, honey helps maintain a nutritional balance for human health. These physicochemical parameters are vital for determining the quality, authenticity, and safety of honey (Bogdanov 2011).

Among honey compositions, HMF and diastase are key quality control measures used to assess the quality, freshness, overheating, and storage conditions of honey (Hasan 2013, Korkmaz and Küplülü 2017, Sajid et al. 2019, Tosi et al. 2002, Tosi et al. 2008, Yilmaz and Küfrevioğlu 2001). According to International Standards, the maximum level of HMF content is 40 mg/kg, whereas the level of diastase content is greater than 8 DN (Codex Alimentarius 2001). According to FSSAI (FSSAI 2019), honeys from tropical regions have a maximum HMF content limit of 80 mg/kg and a diastase level greater than 3 DN.

HMF is produced by the breakdown of fructose and glucose in acidic medium. HMF is present in very small amounts in fresh honey samples, but it increases during heating or storage process (Kamboj et al. 2024, Manickavasagam et al. 2024). A significant increase in HMF level with higher temperatures and longer storage duration stated by earlier research (Kamboj et al. 2024, Manickavasagam et al. 2024, Kamboj et al. 2019, Nanda et al. 2006, Visquert et al. 2004).

Diastase is a thermally stable enzyme secreted by the hypopharyngeal gland of worker bees, able to convert starch into maltose for the production of honey (Bogdanov 2011). The diastase activity is high in fresh honey but reduces during the heating and storage processes (Korkmaz and Küplülü 2017, Kamboj et al. 2024). In the honey samples of *A. mellifera*, 27.68% reduction of diastase number were observed when heated to 65^0C (Sawarkar 2023b). In another study, honey samples heated at 55^0C for 3-15 min showed a 61-70% loss of diastase activity after 23 weeks of storage (Cervantes et al. 2000), while Yilmaz and Frevüoúlu (2001) also reported a 27% decrease in diastase activity.

Invertase is a key enzyme in honey that catalyses the conversion of sucrose to glucose and fructose (Bogdanov et al. 2008). Honey resists fermentation because of its high sugar content, which is caused by invertase activity. Invertase activity is a key indicator for assessing honey freshness and identifying heat exposure (Kamboj et al. 2024). Compare to diastase, invertase is a more reliable honey quality parameter due to its greater sensitivity to temperature and storage conditions (Julika et al. 2022, Sahin et al. 2020).

According to Hasan (2013), reduction of invertase number from 3.11 to 10.7% after three months of storage and 60.21% to 70.5% after six months were observed in three honey samples from Iraq. Invertase activity dropped from 24.31 to 3.88 IN after 6 hours of storage at temperatures over 65^0C (Sahin et al. 2020). Similar result also stated by Karabournioti and

Zervalaki (2001) where invertase activity was reduced by approximately 95% for pine, helianthus, cotton, and thymus honey and 85% for orange honey when heated at 75^{0}C for 24 h. The factors influencing honey quality are well documented in the literature, especially the effects of heating and extended storage on HMF and enzymes as diastase, glucose oxidase, and invertase (Hasan 2013, Kamboj et al. 2019, Julika et al. 2022, Sahin et al. 2020, Karabournioti and Zervalaki 2001).

Dwarf honeybees (*Apis florea*) are widely distributed in agroforest, urban, and rural areas. Honey has been used as a food and medicinal product in tribal and rural communities and has become an important source of income for many rural areas. Analysis of different physicochemical parameters in *Apis florea* honey has been extensively studied by earlier researchers (Al-Ghamdi et al. 2019, Balasubramanyam 2011, Iftikhar et al. 2011, Sajid et al. 2023, Taha et al. 2021). Sawarkar (2023a) was reported about the physicochemical properties of *Apis florea* honey in North Maharashtra. But, no any information on the effect of storage temperature in *Apis florea* honey has been observed in Jalgaon district, North Maharashtra. Hence, the aim of this study was to evaluate the effect of heating and storing on the concentration of HMF, diastase, and invertase in honey of *Apis florea* in the Jalgaon district of North Maharashtra, India.

2. MATERIALS AND METHODS

2.1 Sample Collection

For this study, 10 honey samples of *A. florea* were collected mainly from agroforestry areas in the Jalgaon district, North Maharashtra, India during the year April 2022 to September 2024. All the honey samples were fresh, unheated, and naturally pure, collected directly from tribes and beekeepers. After collection, the samples then filtered through a fine cloth and stored in airtight glass containers at room temperature. 15 aliquots with 20 g of each honey samples were prepared from the stock. Then all aliquots were placed in a water bath at 25^{0}C, 35^{0}C, 45^{0}C, 55^{0}C, and 65^{0}C for 1, 3, and 5 h. After heating treatment, all the aliquots were cooled and stored at room temperature for further analysis.

2.2 Determination of HMF

The HMF content of honey was measured using the spectrophotometric method of the International Honey Commission (2009). In a 50 mL volumetric flask containing 5 g of honey sample was dissolved in 25 mL of distilled water. Then 0.5 mL of Carrez I solution, followed by Carrez II (0.5 mL), was added, and the volume was made up to 50 mL with distilled water. The solution was filtered through a filter paper, and the first 5 mL of the filtrate was discarded. Next, 5 mL filtrate solution was pipetted into two clean and dry test tubes. Five milliliters of 0.2% sodium bisulfite solution was added to one test tube, and 5 mL of pure water was added to another test tube. A UV-visible spectrophotometer was used to measure the absorbance of the solutions at 284 and 336 nm, and the results are expressed in mg/kg.

2.3 Determination of Diastase Activity

Diastase activity in the honey samples was determined according to the International Honey Commission (2009). 10 g of honey samples weighed in a 50 mL beaker and dissolved completely in 15 mL of distilled water and 5 mL of acetate buffer. Then, 3 mL of NaCl was added and the solution was diluted to 50 mL with distilled water.

The starch solution was calibrated using an iodine solution at 660 nm. Pipette 10 mL of honey and starch solution into two separate 50 mL beakers. Both the solutions were heated at 40^{0}C. After 15 min, 5 mL of starch solution was added to the honey solution, mixed thoroughly, and the stopwatch was started. At periodic intervals, an aliquot was taken every 5 min and 5 mL of iodine solution was added rapidly. Then, 11 mL of distilled water was added to each solution, mixed well and absorbance was immediately recorded at 660 nm and the results are expressed in DN.

2.4 Determination of Invertase Activity

Determination of invertase activity in honey samples, analyze by spectrophotometric method (International Honey Commission 2009). Placed 5 g of honey in a 25 mL volumetric flask and added phosphate buffer solution (0.1 M, pH 6.0) and was filled to the mark. Then 5.0 mL of substrate solution (0.02 M p-nitrophenyl-α-D- glucopyranoside) was added to a test tube and incubated in a water bath at 40^{0}C for 5 min before adding the honey solution.

Added 0.50 mL of honey solution to the substrate solution, mixed thoroughly, and incubated at 40^0C. After exactly 20 minutes, add 0.50 mL of the reaction-terminating solution (3 M tris-(hydroxymethyl) aminomethane, pH 9.5) was mixed properly. For the blank, the substrate solution (5.0 mL) was incubated at 40^0C for 5 min. After 5 minutes, add 0.50 mL of the reaction-terminating solution, mix well, and then add 0.50 mL of the honey solution. The solutions were cooled to room temperature as quickly as possible, and the absorbance of the sample solutions was measured at 400 nm using a UV-VIS spectrophotometer. Readings were taken after 15 min and completed within one hour. The absorbance of the blank was then subtracted from that of the sample solution (ΔA400). The invertase activity of the honey samples was determined by multiplying the absorbance by a factor of 21.64 and expressed as the invertase number (IN).

2.5 Statistical Analysis

For each honey sample, HMF, diastase, and invertase activities were determined in triplicate. Data obtained from the honey samples were compared using a t-test. Differences between mean values were considered significant at $P < .05$. Descriptive statistics were used to analyze the data generated through the survey.

3. RESULTS AND DISCUSSION

Honey samples of *A. florea* obtained from the Jalgaon district of the North Maharashtra region were examined. All honey samples were determined and showed variations in HMF, diastase, and invertase content, as summarized in Table 1 and Figs. 1, 2 and 3.

3.1 Evaluation of HMF

In the present study, the HMF content in the examined honey is 33.16 ± 0.93 mg/kg which remains within the International standard (<40 mg/kg) reported by Codex Alimentarius (2001) (Table 1). The HMF levels of honey samples were significantly increased after 1, 3, and 5 h of storage at fixed temperatures (Fig. 1). HMF contents at storage durations of 5 h at specified temperatures of 25^0C, 35^0C, 45^0C, 55^0C and 65^0C were 33.55 (1.15%), 34.98 (5.49%), 37.82 (14.1%), 43.14 (30.1%) and 48.21 mg/kg (45.4%), respectively, indicating that HMF activity increases with temperature and storage time. At 65^0C, the HMF level of honey samples were higher than 40 mg/kg, as per International standards. Even though it increases, HMF content remain at the acceptable international standard of 80 mg/kg as specified by FSSAI (2019), which is mainly observed in tropical climatic environments.

Table 1. Variation in HMF, diastase and invertase content at different storage temperatures of *A. florea* honey samples

Storage Temperature	Time duration	HMF[a] (mg/kg)	HMF%age[b]	Diastase[a] (DN)	DN %age[b]	Invertase[a] (IN)	IN %age[b]
	0 hr	33.16 ± 0.83		18.69 ± 0.45		16.31±0.62	
25^0	1 hr	33.21 ± 0.41	0.15	18.67 ± 0.58	0.11	16.29±0.48	0.12
	3 hr	33.27 ± 0.68	0.33	18.59 ± 0.23	0.54	16.14±0.74	1.04
	5 hr	33.54 ± 0.82	1.15	18.35 ± 0.12	1.82	16.03±0.61	1.72
	1 hr	33.84 ± 1.17	2.05	18.19 ± 0.28	2.68	16.17 ± 0.56	0.86
35^0	3 hr	34.33 ± 0.76	3.53	17.51 ± 0.44	6.31	15.86 ± 0.37	2.76
	5 hr	34.98 ± 0.82	5.49	16.87 ± 0.25	9.74	15.24 ± 0.62	6.56
	1 hr	34.51 ± 1.04	4.07	17.12 ± 0.52	8.4	15.29 ± 0.27	6.25
45^0	3 hr	35.67 ± 0.57	7.57	16.23 ± 0.45	13.2	14.45 ± 0.52	11.4
	5 hr	37.82 ± 0.76	14.1	15.37 ± 0.51	17.8	13.71 ± 0.44	15.9
	1 hr	36.63 ± 0.78	11.1	16.05 ± 0.38	14.1	13.22 ± 0.21	18.9
55^0	3 hr	40.25 ± 1.17	21.4	14.43 ± 0.49	22.8	12.34 ± 0.30	24.3
	5 hr	43.14 ± 1.36	30.1	12.19 ± 0.50	34.8	11.13 ± 0.19	31.8
	1 hr	41.65 ± 1.36	25.6	12.62 ± 0.33	32.5	11.14 ± 0.22	31.7
65^0	3 hr	45.47 ± 1.22	37.1	09.81 ± 0.38	47.5	08.73 ± 0.17	46.5
	5 hr	48.21 ± 1.09	45.4	07.49 ± 0.21	59.9	04.55 ± 0.14	72.1
Standard values		Codex A. (<40 mg/kg) and FSSAI (<80 mg/kg)		Codex A. (>8 DN) and FSSAI (>3 DN)		Codex A. and FSSAI IN ≥ 10	

Results are expressed as Means ± SD.

[a] *The analyses were performed in triplicate.*

[b] *Average percentage calculated by comparing normal value with storage duration at 25^0C, 35^0C, 45^0C, 55^0C, and 65^0C.*

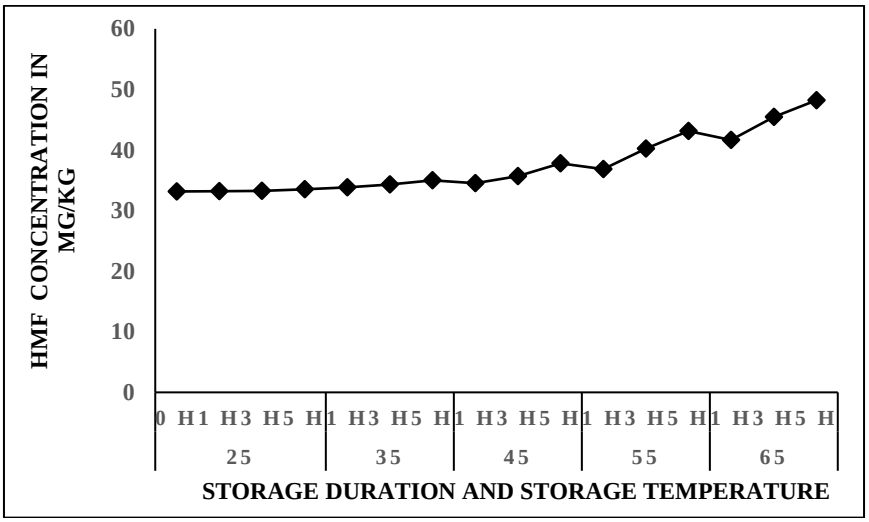

Fig. 1. The HMF content during storage duration and temperature measured in mg/kg

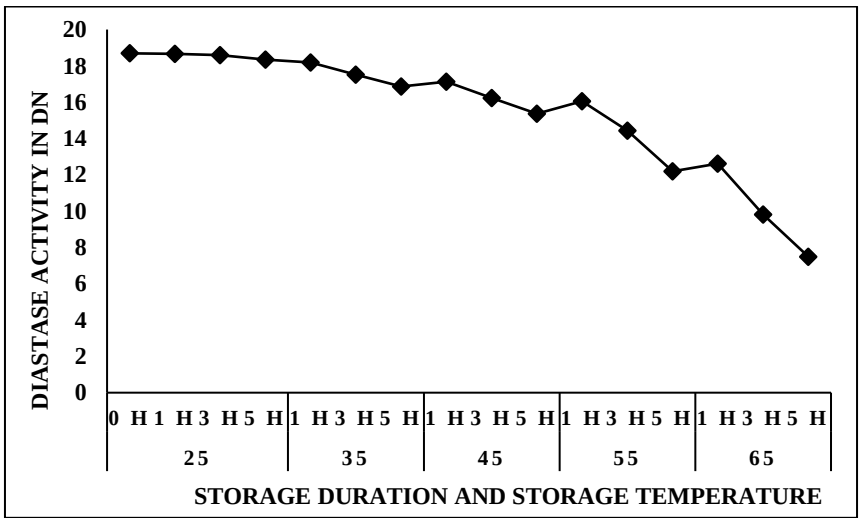

Fig. 2. The diastase activity during storage duration and temperature measured in DN

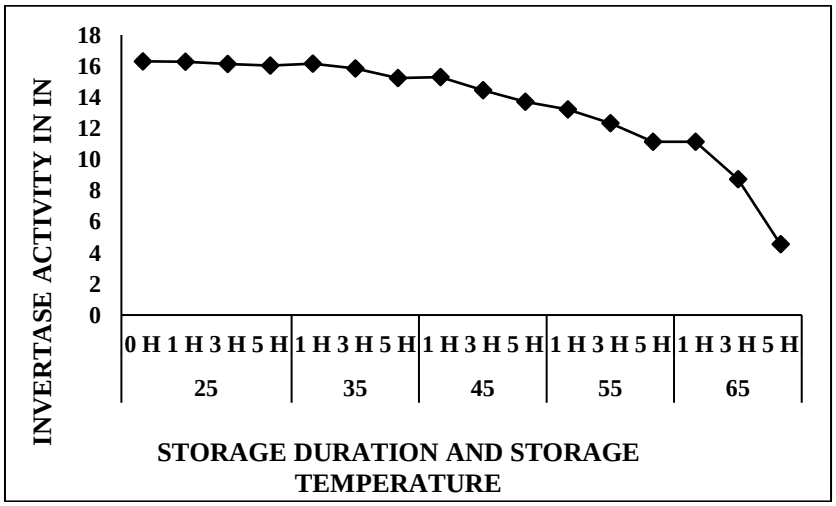

Fig. 3. The invertase activity during storage duration and temperature measured in IN

HMF concentration of honey samples were increased from 6.79 to 55.11 mg/kg after 5 hours of storage duration from 45^{0}C to 90^{0}C (Rababah et al. 2024). According to Al-Ghamdi et al. (2019), after 30 minutes of exposure to 80^{0}C, the HMF content of *A. mellifera* honey was increased from 3.78 to 16.30 mg/100 g, and that of *A. florea* honey rised from 3.17 to 7.41 mg/100 g. Turkut et al. (2018) also observed that three honey samples of different origins (multifloral, honeydew, and chestnut) heated at 50^{0}C, 70^{0}C, and 80^{0}C for period ranging from 0 h to 48 h, resulted in a significant increase in HMF formation and enhanced antioxidant properties.

The observed changes in HMF values across the samples emphasize honey's sensitivity to the duration and intensity of heating were also reported by many researchers (Kamboj et al. 2024, Manickavasagam et al. 2024, Kamboj et al. 2019, Nanda et al. 2006, Visquert et al. 2004). HMF content is not only influenced by heat treatment and storage, but also by many physicochemical factors as floral origin, pH, moisture content, electrical conductivity, free acidity, and sugar profile (Julika et al. 2022, Braghini et al. 2020, Raweh et al. 2022).

3.2 Evaluation of Diastase Activity

Diastase, an enzyme present in honey which might help indicate the freshness and quality of honey because of its heat resistance. The mean diastase value in fresh honey samples of *A. florea* at 25^{0}C was 18.69±0.45 DN, as illustrated in Table 1. All analyzed honey samples complied with the regulatory standards of diastase activity set by FSSAI (2019) and Codex Alimentarius (2001), with values not less than 3 and always greater than 8 DN.

Diastase activity in honey samples significantly decreased after storage for 1, 3, and 5 h at fixed temperatures of 25^{0}C, 35^{0}C, 45^{0}C, 55^{0}C, and 65^{0}C (Fig. 2). The mean value of diastase was 18.69 DN at 25^{0}C which decreased to 7.49 DN (65^{0}C) during the storage duration, indicates that decrease in diastase activity was closely associated with the increase in storage temperature. The results showed statistically significant differences ($P < .05$) in the storage temperature and storage duration. The reduction rate of diastase activity remains highest after 5 hours of storage at temperatures of 55^{0}C and 65^{0}C is 12.19 DN (34.8%) and 7.49 DN (59.9%), shows that storage duration significantly affects diastase content.

According to Cervantes et al. (2000), honey samples heated at 55^{0}C for 3-15 minutes showed 61-70% loss of diastase activity after 23 weeks of storage. Yilmaz and Frevüoúlu (2001) found an average decrease of 27% in diastase numbers in honey samples after one year of storage at 20±5°C, with values decreasing from 14.6 to 10.7. During transient heating, at heating times between 120 and 1200 s, the reduction in diastase activity increased with temperature from 60^{0}C to 90^{0}C, and at 100^{0}C, it was reduced to zero (Tosi et al. 2008). In honey from *A. mellifera*, diastase activity decreased by an average of 11.33% with a temperature drop to 15^{0}C, whereas an increase to 65^{0}C led to an average reduction of 27.68% (Sawarkar 2023b). Therefore, it was concluded that heat treatment at higher temperatures causes a decrease in diastase activity, which may affect the quality and reduce the shelf life of honey. Similar findings have shown that variations in diastase levels in honey samples may be influenced by prolonged honey storage duration, processing, and harvesting time (Korkmaz and Küplülü 2017, Nanda et al. 2006, Sajidet al. 2023, Sahinler 2007, Turhan 2008). In addition, earlier studies have shown that diastase is significantly positively correlated with moisture, pH, electrical conductivity, fructose, and glucose (Kamboj et al. 2019, Raweh et al. 2022).

3.3 Evaluation of Invertase Activity

Invertase enzyme plays a crucial role in honey production by breaking down sucrose into glucose and fructose and increasing the sweetness and stability of honey. Given the importance of invertase, this study was designed in accordance with storage duration and temperature, and the results for *A. florea* honey samples are summarized in Table 1.

The mean invertase content of fresh honey samples was 16.31 IN at room temperature (25°C), which is higher than the minimum invertase number of 10 IN reported by Codex Alimentarius (2001) and FSSAI (2019). In this study, honey samples storing at 25^{0}C, 35^{0}C, 45^{0}C, 55^{0}C, and 65^{0}C temperatures with stoage duration of 1, 3 and 5 hours are significantly affects invertase activity (Table 1). The reduction in invertase number at storage temperatures of 25^{0}C, 35^{0}C, 45^{0}C, 55^{0}C, and 65^{0}C was approximately 1.72%, 6.56%, 15.9%, 31.8%, and 72.1%, respectively. After 1, 3, and 5 h at 65^{0}C, the invertase number decreased steeply to 11.14, 8.73 and 4.55 IN (72.1%), respectively

(Fig. 3). The invertase number decreased to (4.55 IN) after 5 h at 65^{0}C. The collaborative effects of temperature and storage duration on invertase activity were significant ($p < .05$).

In the conventional honey extraction process, 1 h was determined to be the ideal duration, with the optimal temperature set to 24^{0}C. This condition best maintains the active form of the invertase. All honey samples exposed to high temperatures and extended storage times showed a significant reduction in invertase levels, indicating that invertase is highly sensitive to high temperatures and prolonged storage. According to Hasan (2013), the invertase number decreased by 3.11 to 10.7% in honey samples during the first three months, and by 60.21%-70.5% after six months of storage. Sahin et al. (2020) found that as the extraction time increased, invertase activity decreased in all honey samples, and with values ranging from 3.884 IN to 24.313 IN. Significant enzyme inactivation was observed only after prolonged exposure, which required more than 6 h and temperatures above 65^{0}C. Karabournioti and Zervalaki (2001) reported that honey samples (Pine, *Thymus, Cotton, Helianthus*, and orange) from different origins in Athens were heated at temperatures of 35^{0}C, 45^{0}C, 55^{0}C, 65^{0}C, and 75^{0}C for 24 h. Invertase activity decreased with increasing temperature, showing a reduction of approximately 95% for pine, helianthus, cotton, and thymus honey and 85% for orange honey.

In addition to storage temperature and duration, invertase activity increases with higher pH, electrical conductivity, and free acidity at a constant temperature (Kamboj et al. 2024, Kamboj et al. 2019, Qamer et al. 2009, Qamer et al. 2013). The level of invertase may also depend on factors such as floral season, nectar origin, nectar concentration, and sources of nectar, which provide the substrate for converting sucrose into glucose and fructose. Invertase activity is an important indicator of honey freshness, thermal treatment, and fermentation.

4. CONCLUSION

Honey produced from *A. florea* stored at five fixed temperatures (25^{0}C, 35^{0}C, 45^{0}C, 55^{0}C, and 65^{0}C) for 1, 3, and 5 h showed significant variations in the amounts of HMF, diastase, and invertase. The results indicate that heating temperatures of 55^{0}C and 65^{0}C had adverse effects on the concentration of HMF, diastase, and invertase activity compared to honey treated at 25^{0}C, 35^{0}C, and 45^{0}C. The study observed an increase in HMF concentration with longer storage times and higher temperatures. Despite this increase, HMF levels generally remained within the acceptable international standard of 80 mg/kg, as specified by FSSAI (2019), which aligns with observations typically made in tropical climatic conditions. As an important quality marker, HMF is used for the evaluation of honey freshness, the effects of overheating, and how well it is stored. Invertase activity serves as a more sensitive indicator of honey quality than diastase activity because it degrades more readily with heat exposure. While diastase activity is commonly included in honey quality assessments to evaluate storage conditions, invertase may be a superior quality indicator because of its higher susceptibility to heat degradation. The enzymatic behavior, characterized by a marked decrease in diastase and invertase activity, indicates that storage or processing temperatures should not exceed approximately 45^{0}C for long-term application to preserve enzymatic integrity. This study can help beekeepers to understand how proper storage conditions after harvest can protect honey quality and keep it fresh for better marketing.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author is hereby declared that no any propagative AI-technology as Large Language Models and Text-to-image generators have been used throughout preparing and editing this article.

DECLARATION

The material for research i.e. honey was collected directly from beekeepers and tribes for further analysis in the laboratory. This is an observational study. The Institutional Research Ethics Committee, BP Arts, SMA Science & KKC Commerce College, Chalsigaon Dist- Jalgaon (India) has confirmed that no ethical approval is required.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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