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Combating rancidity in pearl millet flour: Assessing the efficacy of physical treatments on lipoxygenase activity

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Abstract
Despite being a rich source of nutrition, with 9-11% protein, 6% lipid, and essential micronutrients like iron and zinc, as well as providing 361 kcal/100 gm of energy, pearl millet flour is not widely consumed due to its propensity to develop rancidity within a short storage period (less than 30 days). This rancidity is primarily enzymatic, involving lipase, lipoxygenase (LOX), polyphenol oxidase, and peroxidase. Lipase generates free fatty acids from lipids, which serve as substrates for LOX. LOX catalyzes the formation of hydroperoxides, which further degrade into aldehydes, ketones, and polymers, causing off-odors and rancidity. This study focused on the LOX 2 isoform, chosen for its substrate specificity and hydroperoxide production, to evaluate different treatments aimed at mitigating flour rancidity. Various physical treatments were applied to assess LOX 2 activity, including dry heat, microwave heat, vacuum packaging, and a combination of microwave and vacuum treatments at different storage intervals (0, 10, 20, and 30 days). Results indicated that the Dhanshakti genotype exhibited the highest LOX activity (39.75 U/mg) on day 10, while Pusa Purple 1 showed the lowest (30 U/mg). A dry heat treatment (90 °C for 2 minutes) followed by 4°C incubation significantly reduced LOX activity in Dhanshakti (28.95 U/mg). All genotypes demonstrated reduced LOX activity compared to the control, with Dhanshakti showing a significant decrease (14.17 U/mg) at day 30. Vacuum treatment effectively reduced LOX activity in the WGI-100 genotype (17.76 U/mg), followed by Pusa Purple 1 (20.35 U/mg) and Dhanshakti (26.8 U/mg). The combined microwave and vacuum treatment further reduced LOX activity, especially in Dhanshakti (12.23 U/mg), followed by WGI-100 (16.34 U/mg) and Pusa Purple 1 (19.77 U/mg).

Keywords: Rancidity, lipoxygenase, microwave, decortication, polyunsaturated fatty acid

Introduction
Pearl millet (*Pennisetum glaucum* L.), a C4 cereal crop from the Poaceae family, is a versatile, cross-pollinated plant with a short life cycle and a genome size of 1.79 GB (Varshney *et al*., 2017, Nature Biotechnology) [22]. This crop boasts a protein content ranging from 7.3% to 13.86% and features a well-balanced amino acid profile. It is rich in vitamins such as Vitamin A, B1, B2, B3, and folic acid, with fat content varying between 4.36% and 7.11%. Notably, pearl millet has superior fat digestibility compared to other cereals, containing up to 75% unsaturated fatty acids. These include omega-3 fatty acids like oleic, linoleic, and linolenic acids, and also saturated fatty acids such as palmitic and stearic acids (Muthamilarasan *et al*., 2016) [13].
The nutritional value of pearl millet is impressive, offering 361 kcal per 100 grams and supplying essential micronutrients like iron and zinc, along with significant antioxidant properties (Vanisha *et al*., 2011) [21]. This robust nutritional profile makes pearl millet superior to other cereals like wheat, rice, sorghum, and maize. In India, pearl millet is traditionally used to make roti, and various marketable products such as biscuits, dhokla, cakes, noodles, and dalia. Beyond its excellent nutritional value, pearl millet offers significant health benefits. It is beneficial for individuals with diabetes (Kam *et al*., 2016) [9] and celiac disease (Shivhare *et al*., 2017; Sarita *et al*., 2016) [19, 18].
There are two types of lipid oxidation contributing to the development of off-odour in pearl millet flour.

The first mechanism is enzymatic rancidity caused by the Lipase enzyme which results in the release of free fatty acids and lipoxygenase which catalyses oxidation of free fatty acid for off flavour generation. Polyphenol oxidase causes enzymatic browning which results in the generation of bitter compounds. The second mechanism is oxidative rancidity, caused by the lipoxygenase activity on free fatty acids and converts into hydro-peroxides and further through peroxide activity into aldehydes, ketones, polymers of peroxides, etc., (Eskin and Przybylski, 2001) [3] which is responsible for off-odour development. Due to the above reason, pearl millet flour can only be stored for a short period of time which is the main drawback for its utilization.
Lipoxygenase (LOX, EC 1.13.11.12) is an iron-containing dioxygenase enzyme that catalyzes the oxidation of polyunsaturated fatty acids and esters into hydroperoxides (Rodrigo *et al*., 2006) [16-17]. Both plant and animal LOX enzymes contain a 100-150 amino acid N-terminal C2-like domain, which interacts with free fatty acids (Offenbacher *et al*., 2018) [14]. There are multiple isoenzymes of LOX in plant tissues, such as LOX1, LOX2, LOX3, and LOX4, each differing in properties like pH optimum and isoelectric point. Their activity is enhanced by Ca2+ and Mn2+ but inhibited by Na+, Zn2+, and K+ (Babitha *et al*., 2004; Ratachatachaiyos and Theerakulkait, 2009) [15]. Antioxidants like vitamins E and C, BHA, and BHT are potent LOX inhibitors (Theerakulkait and Barrett, 1995) [20]. LOX also contributes to the formation of volatile flavor compounds in fruits and vegetables, creating "fresh" and "green" sensory notes (Rodrigo *et al*., 2006) [16-17].
Based on the rancidity issue in pearl millet and lipoxygenase for being the primary culprit for oxidation the current study has been undertaken to understand different physio-chemical method that reduces the off-flavour generation in pearl millet

Materials and Methods

Three pearl millet varieties (Dhanshakti, WGI-100 and Pusa Purple 1) were provided from
Division of Genetics, IARI, New Delhi and AICRP on pearl millet, Jodhpur. Grains were
grounded using a mini miller machine to 100 mesh size particles. Powdered samples were
stored in air-tight amber-colored bottles at room temperature (RT) for various physiological and biochemical analyses.

Dry heat treatment

The pearl millet grains were kept in hot air oven at 90 °C for 2 min. After incubation the grains were kept at RT for 30 min and used for LOX 2 and peroxide value estimation. The above treatment was modified by storing grains at 4°C immediately after the dry heat treatment (chilling) and used further for LOX 2 and peroxide value estimation.

Microwave treatment

Microwaves are high penetrating radiation and raise temperature in sec. The pearl millet
grains were heated in microwave for 30 sec and acid value and LOX 2 activity was estimated in these samples.

Decortication

Decortication i.e. removal of pericarp layer (Lipases are located mainly in pericarp) was
carried out using rice mill peeler in Dhanshakti and this sample was named as decorticated
Dhanshakti and taken as an additional sample for further studies.

Vacuum treatment

Vacuum packing of flour is one of the approaches to arrest lipid oxidation. Pearl millet flour was packed using vacuum machine SWIFT PACK and stored for further biochemical analysis.

Enzyme extract preparation

Flour was prepared using grinding machine. Take 1gm of finely powdered flour in oakridge tube and dissolve it in 10 mL of 0.2M Phosphate buffer (pH 7.5). Vortex it properly for 60 sec to dissolve uniformly in buffer. Centrifuge it at 10000 rpm for 30 min. Take supernatant and filter it through muslin cloth. Transfer supernatant to another oakridge tube and keep it at 4°C for further analysis.

Protein estimation

Concentration of protein was estimated using Bradford method. Take test tube and add 2 ml of Bradford reagent (soluble dye Coomassie Brilliant Blue G250. Add 990 µl water followed by 10 µl of protein extract. Keep it in dark for 10 min. Take absorbance at 595 nm.

Lipoxygenase (LOX 2) Enzyme Activity Assay

Substrate (linoleic acid) was prepared by the method of Surrey (1964). Tween 20, 50 µl, was dissolved in 5 ml of double distilled water, and 35 µl of linoleic acid was added drop by drop. The contents were thoroughly mixed so as to disperse the acid into a fine emulsion. Then 600 µl of 0.5N NaOH was added and the mixture once again agitated until a clear transparent solution was obtained. Make up the volume 100ml with 0.2 M potassium phosphate buffer, pH 6.8 and maintain pH up to 6.6. The reaction mixture in a final volume of 2.66 mL contained 2.575 mL of 0.05 M acetate buffer (pH 4.2), 60 µl of 7.5 mM linoleic acid and 0.25 mL of enzyme extract. The reaction was started by addition of the enzyme and progress of the reaction was monitored by recording increase in absorbance at 234 nm for 2 min against blank. To calculate LOX 2 activity, extinction coefficient used was 25 mM^{-1} cm^{-1} and expressed as µmol min -1 g -1 DM of seed tissues. One unit of LOX activity is defined as 1µmole of hydro peroxide formed per minute at 25 ºC.

Results

Fat content was determined in experimental genotypes and it was found maximum in purple variety (5%) followed by 4.6% in corticated Dhanshakti variety and 4.5% in WGI-100 and least in decorticated Dhanshakti variety (Fig. 1)

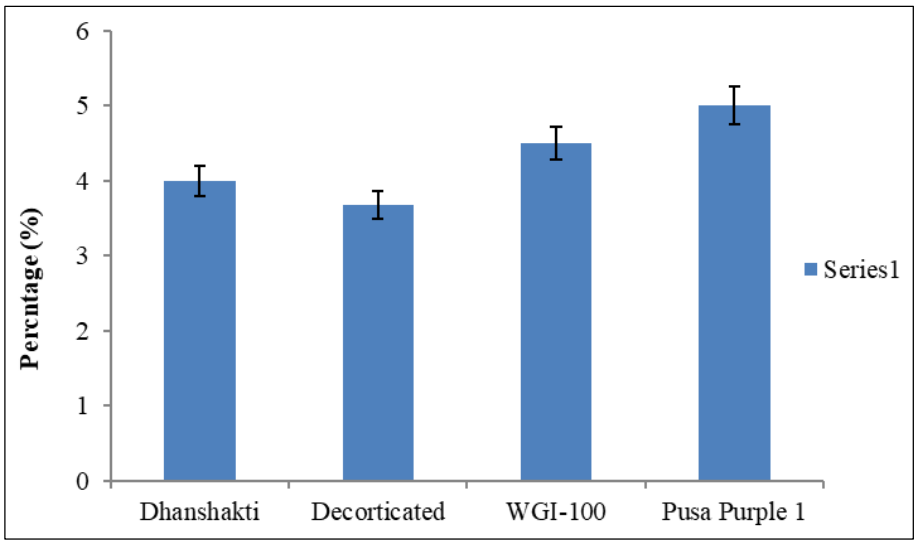

Fig 1: Representing fat content in different variety of pearl millet

Effect of storage on LOX2 activity

In order to analyze the activity of LOX 2, three different genotypes of pearl millet were selected and flour sample of each variety was used for the analysis. LOX activity was measured at different storage periods *viz*; 0, 10, 20, and 30days. The results showed increasing trends in the activity of LOX 2 in flour stored upto 10 days. The activity of LOX 2 was found to be significantly higher at 10th day of storage (39.7589 U/mg) in case of Dhanshakti variety accounting up to 12% increase in activity as compared to 20th and 30th days of storage where it showed 35% and 38% reduction in activity respectively. Whereas in case of WGI-100 variety similar trend was observed i.e. increased LOX 2 activity up to 10 days however at 20th day (23.26 U/mg) and 30th day (19.3 U/mg) of storage, the reduction in LOX 2 activity was found to be reduced to 14% and 20% respectively. The same trend was observed in case of purple variety Pusa Purple1 where LOX activity was found highest on 10th day (30.004 U/mg) of storage however at 20th day (26.81 U/mg) and 30th day (19.7 U/mg) of storage reduction in LOX 2 activity was observed by 10% and 29% respectively (Fig. 2).

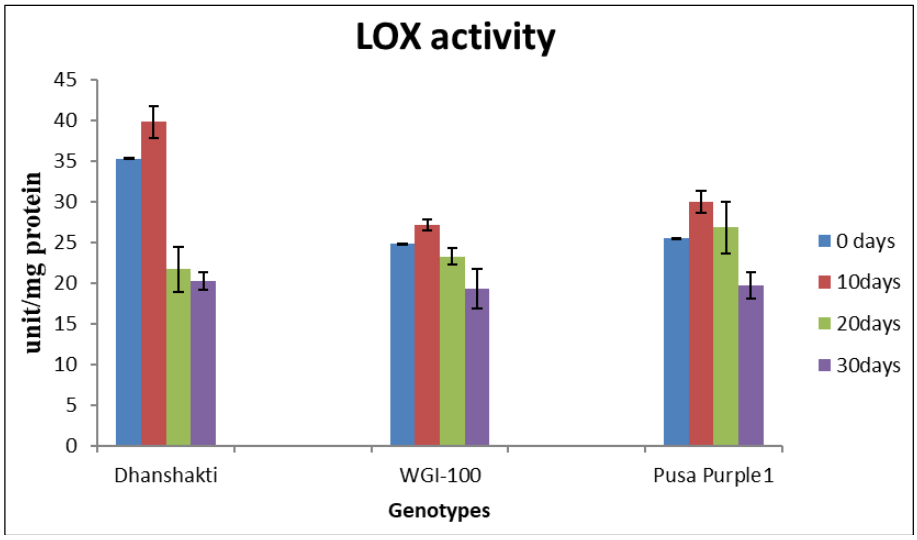

Fig 2: Representing effect of storage on LOX2 activity in different variety of pearl millet

Effect of dry heat treatment

Based on above data temperature and time period was selected since Dhanshakti genotype showed higher LOX activity therefore it was further selected for dry heat treatment at 90°C for 2 minutes. Among all the rancidity related enzymes i.e. lipase, LOX, polyphenol oxidase, and peroxidase, lipase is most resistant to heat. Boli *et al*. (2016) showed inactivation of lipase and LOX below 90°C by giving SIR (Short Infrared Radiation) treatment and found that it not irreversibly destroys the lipase and LOX activity so we have chosen 90 °C for 2 min to check the activity of LOX 2. In our study Dhanshakti grain were treated at 90 °C for 2 minutes followed by incubation for 30 minutes at room temperature and flour was prepared to analyze LOX 2 activity. The result showed no significant decrease in activity of enzyme (34.31 U/mg) as compared to control (36.82 U/mg). However heat treatment incubation of flour at 4 °C for 15 minutes showed significant reduction in LOX activity (28.95 U/mg) which was accounting for 23% reduction in LOX 2 activity as compared to 7% reduction in former experiment. Thus, our result showed that heat treatment of grain at 90 °C for 2 minutes followed by incubation at 4 °C for 15 minutes was found to be better treatment to reduce the activity of LOX 2 (Fig. 3).

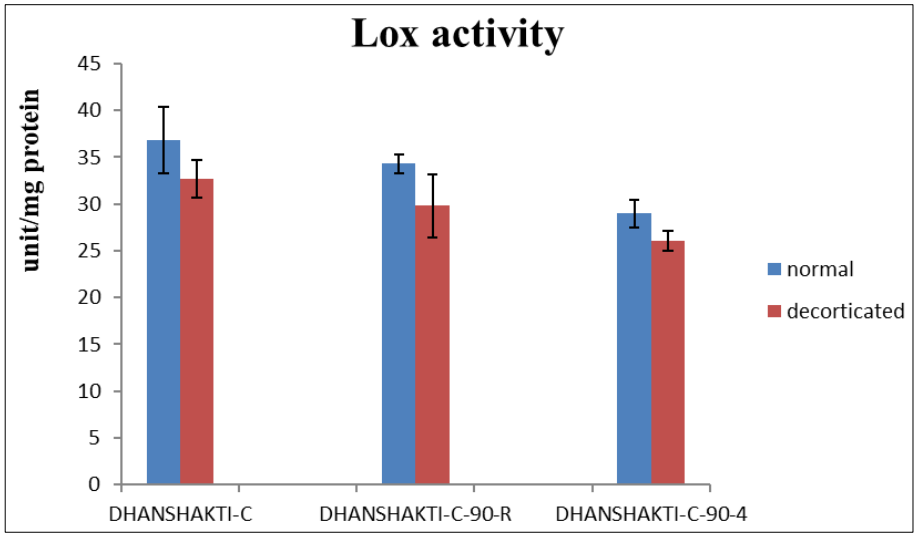

Fig 3: Representing effect of dry heat treatment on LOX2 activity in Dhanshakti variety of pearl millet

Effect of moist heat treatment

Different time period were selected to give moist heat treatment i.e. 10, 20 30 40 60 second and standardized the time period for 30 second based on the no colour change in the grain. Moist heat treatment was given to grain of all experimental genotypes using microwave (1 mm to 1 m wavelength) for 30 seconds. Flour was prepared and activity was measured. The result showed significant reduction in LOX 2 activity (18.69 U/mg) in Dhanshakti genotype as compared from control (35.27 U/mg) and which was accounting for 35% reduction in activity. Similar kinds of result were observed in purple variety Pusa Purple1 and white variety WGI-100 flour. Increasing trend in activity of LOX 2 was found at 10th day of storage (26.29 U/mg in case of Pusa Purple1 and 20.98 U/mg in case of WGI-100) whereas reduction in activity of LOX 2 was observed at 20th day of storage (21.90 U/mg in case of Pusa Purple1 and but not effective reduction in case of WGI-100) and 30th day (19.24 U/mg in case of purple variety while 17.71 U/mg in case of white variety WGI-100). Thus, our result suggested that moist heat treatment as the most effective method for reduction of LOX 2 activity (Barber and Benedito de Barber, 1980) [1]. This treatment results in even distribution of heat (Roman *et al*., 1989). Further in order to check the effectiveness of moist heat treated flour sample was stored for analysis at 10th, 20th and 30th day. In Dhanshakti variety the result showed increase in trend in activity of LOX 2 at 10th day of storage (21.6 U/mg) and increase was found up to 15%. However significant decrease in activity of LOX 2 was found at 20th day (16.57 U/mg) and 30th day (14.17 U/mg) which was accounting up to 25% and 33% respectively (Fig. 4).

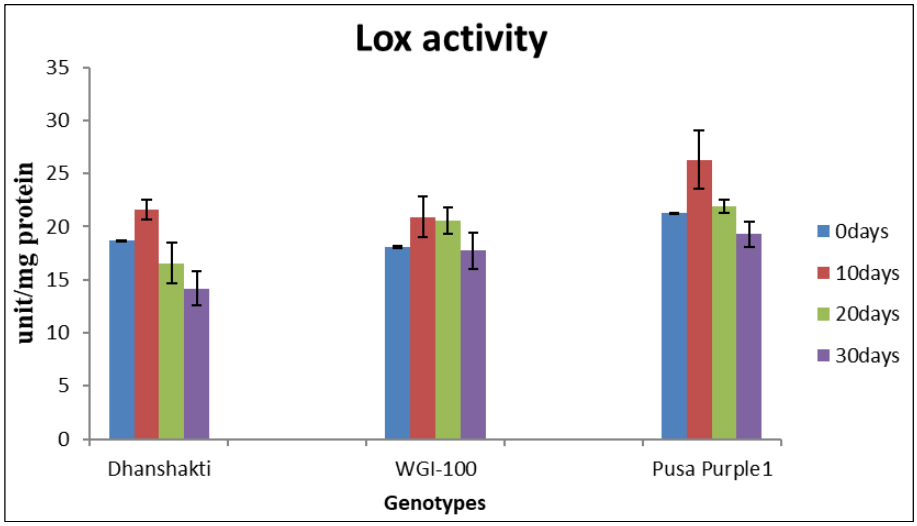

Fig 4: Representing effect of moist heat treatment on lox activity in different variety of pearl millet

Effect of decortication

Decortication is a physical method of treatment to remove the pericarp of seed. Removal of pericarp is an important step as it contains lipase which generates free fatty acid which is the substrate for LOX. The previous results Dhanshakti showed better response to moist heat treatment, based on this decortications treatment was given to Dhanshakti genotype. The result showed significant reduction ($p< 0.05$) in LOX 2 activity (32.71 U/mg) which account for 11% reduction as compared to normal. Further the decorticated variety was stored for 10th, 20th and 30th days. The result showed significant reduction ($p< 0.05$) in LOX 2 activity on 30th day of storage (16.45 U/mg) as compared to 10th (37.45 U/mg) and 20th day (20.11 U/mg). Although this method is not effective, due to loss of lipids which composed of essential fatty acid in outer layer of seed; this method was not proceeded for further analysis (Fig. 5).

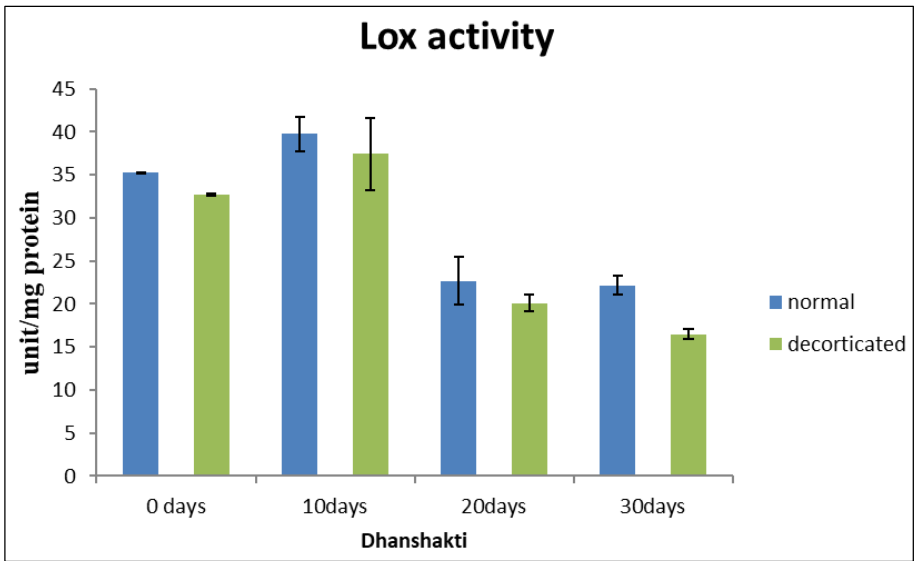

Fig 5: Representing effect of decortication on lox activity in Dhanshakti variety of pearl millet

Effect of vacuum treatment

Vacuum treatment was selected to analyze LOX 2 activity in all genotype as LOX enzyme is sensitive to oxygen. So to carry out all this experiment flour prepared from all the variety was packed and vacuum sealed and kept for 30 days of storage. The result showed significant ($p< 0.05$) reduction in LOX 2 activity (26.87 U/mg in case of Dhanshakti variety, 17.76 U/mg in WGI-100 and 20.35 in case of Pusa Purple 1. However, the decorticated Dhanshakti genotype shows significant ($p< 0.05$) reductions in LOX 2 activity (22.27 U/mg) compared to normal (32.71 U/mg) (Fig. 6).

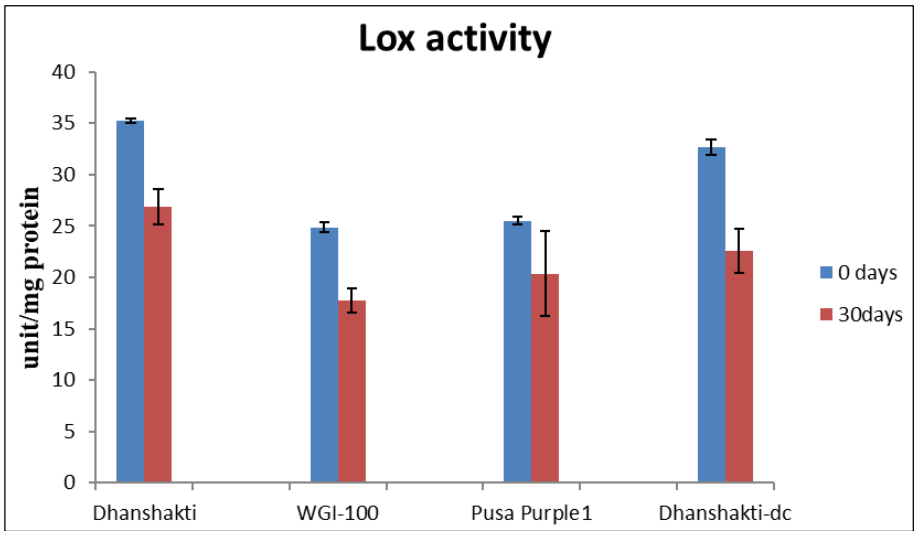

Fig 6: Representing effect of vacuum treatment on lox activity in different variety of pearl millet

Effect of combined treatment of microwave and vacuum

The combined treatment of microwave and vacuum treatment was given to all 3 varieties. The grain of all three varieties was subjected to moist heat treatment using microwave and flour was prepared, packed and sealed under vacuum. The sample was stored up to 30 days. The result showed significant reduction in LOX 2 activity in all variety. Significant ($p< 0.05$) reduction in LOX 2 activity was found in Dhanshakti genotype (12.73 U/mg) followed by WGI-100 (16.34 U/mg) and purple (19.77 U/mg). So, we found that the combination of both microwave and vacuum treatment was more effective as compared to treatment given individually (Fig. 7).

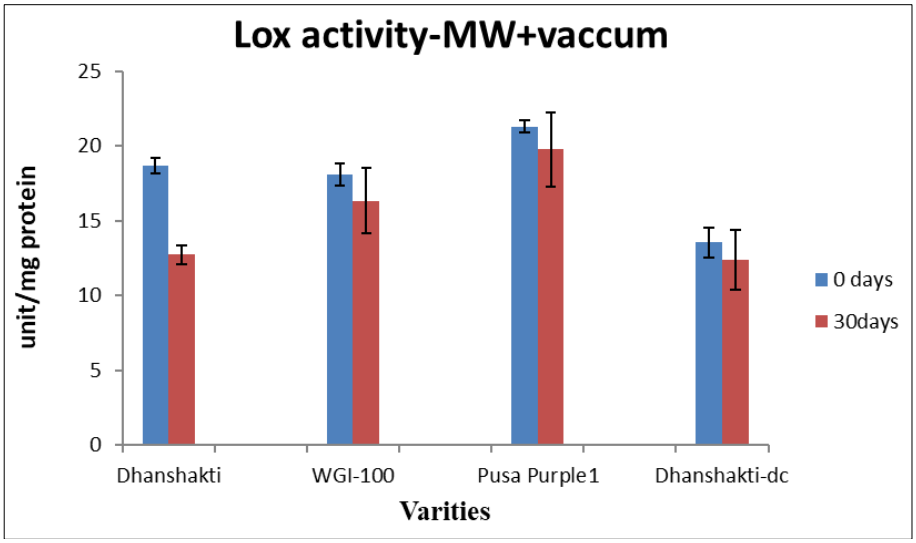

Fig 7: Representing combined effect of microwave treatment and vacuum treatment on lox activity in different variety of pearl millet

Finally, the response of pearl millet genotype was varied to different treatment. Pusa Purple1 genotype showed lowest LOX 2 activity as compared to other two varieties without any treatment. This suggest that inherent property of purple variety contain higher antioxidant as it is evident from dark purple colour of the seed which might hinder the oxidation of free fatty acid. So the inherent lower activity in purple variety was found to be non-stable when it was subjected to different treatment and showed higher LOX 2 activity to different treatment suggesting the protective role of antioxidant in preventing free fatty acid oxidation might be affected due to treatment.

Discussion

The fat content was analysed in experimental genotypes, with the highest observed in the purple variety (5%), followed by the corticated Dhanshakti variety (4.6%), WGI-100 (4.5%), and the lowest in the decorticated Dhanshakti variety. These findings align with Jain (2013) [8] for HHB 94 pearl millet and Cepkova (2014) [2], who reported varying fat content across pearl millet genotypes. In this study, different rancidity parameters were assessed, focusing on LOX activity and peroxide value to evaluate rancidity levels and shelf life across three genotypes. LOX 2 activity was notably higher in the Dhanshakti variety compared to other genotypes, with the lowest activity in the Pusa Purple1 variety. The purple variety is rich in flavonoids, which act as antioxidants and inhibit LOX 2 activity. Over time, LOX 2 activity increased across all genotypes, peaking at 10 days of storage before decreasing at 20 and 30 days, likely due to the initial abundance and subsequent depletion of free fatty acids, the substrate for LOX 2. A significant reduction in LOX 2 activity was observed with high-temperature treatment (90 °C for 2 minutes) followed by incubation at 4°C for 15 minutes, compared to room temperature incubation for 15 minutes. This suggests that the inhibition of LOX 2 activity at higher temperatures may be due to protein denaturation, which was maintained by subsequent cooling at 4 °C, thereby inactivating the enzyme. Mitsuda *et al.* (1976) [12] also reported increased conjugated diene hydroperoxide levels in samples stored at 25 °C, indicating higher LOX 2 activity compared to those stored at 4 °C. Microwave heat treatment was also evaluated for its effectiveness in reducing LOX 2 activity, showing genotypic variation. LOX 2 activity increased in the purple variety, suggesting a loss of antioxidants that prevent the oxidation of unsaturated free fatty acids. The experiment concluded that moist heat treatment is the most effective for reducing LOX 2 activity across all genotypes, as it resulted in significant reductions compared to dry heat treatment. Similar findings were reported by Barber *et al.* (1980) [1], who observed lower LOX activity under moist heat treatment in crops. Roman *et al.* (1989) also found moist heat to be the most effective in reducing rancidity-related enzymes due to its even distribution and strong penetrating power compared to dry heat. Vacuum treatment was also applied to flour prepared from all three genotypes, resulting in significant ($p< 0.05$) reductions in LOX 2 activity: 24% in comparison to non-vacuumed samples, 29% in WGI-100, and 20% in Pusa Purple1. Vacuum treatment was chosen because O2 is a major substrate for LOX catalytic activity (Malekian *et al.*, 2000) [10]. A combined treatment of microwave and vacuum was applied to all three varieties. Grains were subjected to moist heat using a microwave, and the resulting flour was analyzed for LOX activity. The results showed significant reductions in LOX activity: 32% in Dhanshakti grey variety and 10% in both WGI-100 and Pusa Purple1.

Conclusion

From the present study it was found that, Pusa Purple 1 showed minimum activity of LOX 2 among all the experimental genotypes without subjecting to any treatments. Genotype dependent response was observed under microwave treatment, and found most effective in reducing the LOX 2 activity in case of Dhanshakti variety. Although LOX 2 activity was reduced in all the genotypes under vacuum treatment, we found combination of both microwave and vacuum treatment more effective in reducing the LOX 2 activity in all the genotypes. Rancidity of flour (in terms of peroxide value) was found to be significantly reduced under combined treatment of microwave and vacuum in all the genotypes.

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