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Evaluation of different concentrations of lipase on multigrain functional bread and its nutritional analysis

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Abstract
In the present study a successful attempt has been made to formulate a variety of functional breads that combine unique taste and nutritional balance. The strategic approach for the production of light and healthy bread by replacing harmful chemical improves with respective optimized doses of lipase of *P. polymyxa* G7 in order to enhance product quality and earn a natural and clean label status to a variety of breads which are highly sought after by health conscious consumers of modern age. In addition pseudocereal grains like buckwheat, amaranth and rye are used as bread ingredients which contain high range of beneficial compounds for health. Compounds like flavonoids, phenolic acids and vitamins are available in these grains. Therefore incorporation of these grains in bread dough formulation enhances significantly the nutritional quality of baked product.

Keywords: Bread, *P. polymyxa* G7, pseudocereal grains, nutritional quality

1. Introduction
Bread is commonly consumed almost by everyone and is produced from wheat in different types depending on people's taste, facilities available and conditions. Baking capacity depends on the characteristics of flour, industrial procedures and factors, method of production and manufacturing techniques (Rajabzadeh, 1996) [1]. In recent trend, there has been a special significance on using lipases and phospholipases in replacement of chemical emulsifiers like SSL and DATEM in food industry (Moayedallaie *et al*., 2010; Castello *et al*., 1998) [2, 3]. Lipase enzyme is safe to use in food products since it is a natural constituent of wheat and causes no harm as it is removed due to denaturation and does not exist in the final product (Barcenas *et al*., 2002) [4]. This enzyme benefits the final product in different ways such as increase in durability of dough and capacity of gas storage, development of a consistent structure in the nucleus of bread, refinement in its softness, and rise in the bread loaf volume (Olesen *et al*., 2000; Colakoglu and Ozkaya, 2012) [5, 6]. In order to achieve better results and choose the best concentration of lipase in this study, three other levels were investigated in the control group (Without lipase enzyme).

2. Material and Methods
2.1 Lipase source and its characterization
Lipase from *Paenibacillus polymyxa* G7 (MF446912) has been isolated from resins of *Pinus roxburghii*. The lipase production from *P. polymyxa* G7 has been optimized through one variable at a time approach (OVAT) and statistical technique i.e. Plackett-Burman design (PBD) and Response Surface Methodology (RSM). The optimized conditions were used for purification of lipase from *P. polymyxa* G7 through sequential purification steps. The purified enzyme was finally utilized for preparation of multi grain functional bread.

2.2 Functional wheat bread

Ingredient	Amount used
Wheat flour	50g
Yeast	0.5g
Sugar	1g
Salt	0.5g
P. polymyxa G7 purified lipase	0.5, 1 and 1.5 mg (10, 20 and 30 ppm)
Water	25 ml
Commercial yeast/malera	5g (wet weight)

2.2.1 Sets prepared
1. Control without yeast
2. Control with commercial yeast
3. Control with malera
4. Set-A: Bread containing 10 ppm *P. polymyxa* G7 purified lipase with commercial yeast
5. Set-B: Bread containing 20ppm *P. polymyxa* G7 purified lipase with commercial yeast
6. Set-C: Bread containing 30 ppm *P. polymyxa* G7 purified lipase with commercial yeast
7. Set-D: Bread containing 20 ppm *P. polymyxa* G7 purified lipase with malera

2.2.2 Recipe

Ingredients were weighed and mixed properly
↓
The mixture was knead for 10 min to make a smooth paste
↓
Dough left to rest in bulk for 1h 10 min at 28˚C
↓
Baking was carried out for 12 min at 220˚C
↓
Bread quality characteristics were assessed 24h after baking
↓
Cooled loaves were packed in clean zip-lock pouches and stored at room temperature

Preparation of functional wheat bread

2.2.3 Analysis of physico-chemical parameters
2.2.3.1 Baking weight loss (%, Saric *et al*., 2014) [7]
Baking weight loss (BWL) was determined by measuring the bread weight before and after baking where the bread weight was determined as an average value of 8 independent measurements. Baking weight loss was calculated according to the equation given below:

$$\text{Baking weight loss (BWL, in \%)} = \frac{\text{Intial bread weight (g)} - \text{Weight after baking (g)}}{\text{Intial bread weight (g)}} \times 100$$

2.2.3.2 Diameter W (cm)
To measure the diameter (W) of bread. The bread sample was taken and the diameter was measured from different sides in triplicates. The average of the triplicate values divided by three was taken as the final diameter of the bread.

2.2.3.3 Thickness T (cm)
The thickness (T) of the bread was determined in centimeter with the help of a vernier caliper. Thickness was taken by measuring the bread from bottom to top from different angles in triplicates. The average of the triplicate values divided by three was recorded as the final thickness of the bread.

2.2.4 Analysis of compositional parameters
2.2.4.1 Carbohydrates (Sadasivam andManickam, 1992) [8]
The phenol-sulphuric acid method was used to estimate carbohydrates as described by Sadasivam and Manickam, (1992) [8]. To the diluted sample, 1ml of phenol solution [5 % (v/v)] was added and mixed properly in a test tube. Then, 5 ml of 96 % (v/v) sulphuric acid was added and shaken well. The tubes were kept in a water bath at 25-30°C for 20 min, the absorbance was recorded at 490nm and compared with standard curve prepared with glucose. Standard of glucose was prepared. The standard curve was prepared using different concentrations i.e. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of glucose.

2.2.4.2 Crude protein (Ranganna, 2000) [9]
Weighed sample (1.0 g) was digested with concentrated sulphuric acid (25 ml) along with digestion mixture containing 1-2 g of catalyst mixture (K2SO4:CuSO4:SeO2) in 800 ml long neck Kjeldahl digestion flask till it becomes free from carbon. Distilled ammonia (with excess saturated alkali) from the digest was then absorbed in excess of 0.1 N HCl and left over HCl was back titrated against standardized 0.1 N NaOH using methyl red as an indicator. Crude protein content (%) in the sample was then calculated by multiplying percent nitrogen with the factor of 6.25.

$$\text{Nitrogen (\%)} = \frac{\text{(Sample titre} - \text{Blank titre)} \times \text{Normalityof HCl} \times 14}{\text{Weight of sample} \times 1000} \times 100$$

Crude protein (%) = Nitrogen % × 6.25

2.2.4.3 Crude fat content (Folch, 1957) [10]
Dried sample of 5 g was extracted with petroleum ether in Soxhlet extraction apparatus for 6 hr. The ether extract was filtered in pre-weighed beakers. Petroleum ether was evaporated completely from the beakers and the increase in weight of beaker represented the fat content. The fat (%) obtained was estimated as (g fat/ g dry biomass) × 100

2.2.4.4 Free fatty acid determination (Cox and Pearson, 1962) [11]

2.2.4.4.1 Principle
The free fatty acid in the sample was estimated by titrating it against KOH in the presence of phenolphthalein indicator. The acid number is defined as the mg KOH required to neutralize the free fatty acids present in 1g of sample. However, the free fatty acid content was expressed as oleic acid equivalents.

2.2.4.4.2 Materials
1. 1% phenolphthalein in 95% ethanol.
2. 0.1N potassium hydroxide
3. Neutral Solvent: Mix 25mL ether, 25mL 95% alcohol and 1mL of 1% phenolphthalein solution and neutralize with N/10 alkali

2.2.4.4.3 Procedure
1. 1g of sample was dissolved in 50mL of the standard solvent in a 250mL conical flask.
2. Few drops of phenolphthalein were added to the prepared mixture.
3. The contents were titrated against 0.1N potassium hydroxide.
4. The contents of the flask were constantly mixed until a pink color which persists for fifteen seconds was obtained.

2.2.4.4.4 Calculation

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titrate value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of sample (g)}}$$

The free fatty acid was calculated as oleic acid using the equation
1ml N/10 KOH = 0.028g oleic acid.

2.2.5 Sensorial evaluation
Nine point hedonic scale method as given by Amerine *et al*., (1965) [12] was followed for conducting the sensory evaluation of functional white bread. The panel of 10 judges comprising the students of Department of Basic Sciences, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan was selected to evaluate the functional product for sensory parameters such as color, aroma, texture, softness, mouth feel and overall acceptability. The sample was presented to judges and plain water was given to them to rinse their mouth in between the evaluation of samples. No discussion during evaluation was allowed.

2.2.6 Storage/stability
Storage/stability of functional bread was evaluated upto 20 days.

2.3 Functional wheat and rye bread
Wheat flour + Rye was added in the ratio of 25g: 25g and rest of the recipe is same as given in 2.2.2.

2.3.1 Sets prepared
1. Control without yeast
2. Control with commercial yeast
3. Control with malera
4. Set-A: Bread containing 20 ppm *P. polymyxa* G7 purified lipase with commercial yeast
5. Set-B: Bread containing 30ppm *P. polymyxa* G7 purified lipase with commercial yeast
6. Set-C: Bread containing 40 ppm *P. polymyxa* G7 purified lipase with commercial yeast
7. Set-D: Bread containing 30 ppm *P. polymyxa* G7 purified lipase with malera

2.3.2 Recipe: Same as in section 2.2.2.
2.3.3 Analysis of physico-chemical parameters
2.3.3.1 Baking weight loss (%, Saric *et al*., 2014) [7]: Same as in section 2.2.3.1.
2.3.3.2 Diameter W (cm): Same as in section 2.2.3.2.
2.3.3.3 Thickness T (cm): Same as in section 2.2.3.3.
2.3.4 Analysis of compositional parameters
2.3.4.1 Carbohydrates (Sadasivam and Manickam, 1992) [8]: Same as in section 2.2.4.1.
2.3.4.2 Crude protein (Ranganna, 2000) [9]: Same as in section 2.2.4.2.
2.3.4.3 Crude fat content (Folch, 1957) [10]: Same as in section 2.2.4.3.
2.3.4.4 Free fatty acid determination (Cox and Pearson, 1962) [11]: Same as in section 2.2.4.4
2.3.5 Sensorial evaluation: Same as in section 2.2.5.
2.3.6 Storage/stability: Same as in section 2.2.6

2.4 Functional wheat and amaranth bread
Wheat flour + amaranth was added in the ratio of 25g: 25g and rest of the recipe is same as given in 2.2.2.

2.4.1 Sets prepared
1. Control without yeast
2. Control with commercial yeast
3. Control with malera
4. Set-A: Bread containing 30 ppm *P. polymyxa* G7 purified lipase with commercial yeast
5. Set-B: Bread containing 40ppm *P. polymyxa* G7 purified lipase with commercial yeast
6. Set-C: Bread containing 50 ppm *P. polymyxa* G7 purified lipase with commercial yeast
7. Set-D: Bread containing 50 ppm *P. polymyxa* G7 purified lipase with malera

2.4.2 Recipe: Same as in section 2.2.2.
2.4.3 Analysis of physico-chemical parameters
2.4.3.1 Baking weight loss (%, Saric *et al*., 2014) [7]: Same as in section 2.2.3.1.
2.4.3.2 Diameter W (cm): Same as in section 2.2.3.2.
2.4.3.3 Thickness T (cm): Same as in section 2.2.3.3.
2.4.4 Analysis of compositional parameters
2.4.4.1 Carbohydrates (Sadasivam and Manickam, 1992) [8]: Same as in section2.2.4.1.
2.4.4.2 Crude protein (Ranganna, 2009) [9]: Same as in section 2.2.4.2.
2.4.4.3 Crude fat content (Folch, 1957) [10]: Same as in section 2.2.4.3.
2.4.4.4 Free fatty acid determination (Cox and Pearson, 1962) [11]: Same as in section 2.2.4.4
2.4.5 Sensorial evaluation: Same as in section 2.2.5.
2.4.6 Storage/stability: Same as in section 2.2.6

2.5 Functional wheat and buckwheat bread
Wheat flour + buckwheat was added in the ratio of 25g: 25g and rest of the recipe is same as given in 2.2.2.

2.5.1 Sets prepared
1. Control without yeast
2. Control with commercial yeast
3. Control with malera
4. Set-A: Bread containing 20 ppm *P. polymyxa* G7 purified lipase with commercial yeast
5. Set-B: Bread containing 30ppm *P. polymyxa* G7 purified lipase with commercial yeast
6. Set-C: Bread containing 40 ppm *P. polymyxa* G7 purified lipase with commercial yeast
7. Set-D: Bread containing 40 ppm *P. polymyxa* G7 purified lipase with malera

2.5.2 Recipe: Same as in section 2.2.2
2.5.3 Analysis of physico-chemical parameters
2.5.3.1 Baking weight loss (%, Saric *et al*., 2014) [7]: Same as in section 2.2.3.1.
2.5.3.2 Diameter W (cm): Same as in section 2.2.3.2.
2.5.3.3 Thickness T (cm): Same as in section 2.2.3.3.
2.5.4 Analysis of compositional parameters
2.5.4.1 Carbohydrates (Sadasivam and Manickam, 1992) [8]: Same as in section 2.2.4.1.
2.5.4.2 Crude protein (Ranganna, 2009) [9]: Same as in section 2.2.4.2.
2.5.4.3 Crude fat content (Folch, 1957) [10]: Same as in section 2.2.4.3.
2.5.4.4 Free fatty acid determination (Cox and Pearson, 1962) [11]: Same as in section 2.2.4.4.
2.5.5 Sensorial evaluation: Same as in section 2.2.5.
2.5.6 Storage/stability: Same as in section 2.2.6

2.6 Functional wheat and maize bread
Wheat flour + maize flour was added in the ratio of 25g: 25g and rest of the recipe is same as given in 2.2.2.

2.6.1 Sets prepared
1. Control without yeast

2. Control with commercial yeast
3. Control with malera
4. Set-A: Bread containing 20 ppm *P. polymyxa* G7 purified lipase with commercial yeast
5. Set-B: Bread containing 30ppm *P. polymyxa* G7 purified lipase with commercial yeast
6. Set-C: Bread containing 40 ppm *P. polymyxa* G7 purified lipase with commercial yeast
7. Set-D: Bread containing 30 ppm *P. polymyxa* G7 purified lipase with malera

2.6.2 Recipe: Same as in section 2.2.2.
2.6.3 Analysis of physico-chemical parameters
2.6.3.1 Baking weight loss (%, Saric *et al*., 2014) [7]**:** Same as in section 2.2.3.1.
2.6.3.2 Diameter W (cm): Same as in section 2.2.3.2.
2.6.3.3 Thickness T (cm): Same as in section 2.2.3.3.
2.6.4 Analysis of compositional parameters
2.6.4.1 Carbohydrates (Sadasivam and Manickam, 1992) [8]**:** Same as in section 2.2.4.1.
2.6.4.2 Crude protein (Ranganna, 2009) [9]**:** Same as in section 2.2.4.2.
2.6.4.3 Crude fat content (Folch, 1957) [10]**:** Same as in section 2.2.4.3.
2.6.4.4 Free fatty acid determination (Cox and Pearson, 1962) [11]**:** Same as in section 2.2.4.4.
2.6.5 Sensorial evaluation: Same as in section 2.2.5.
2.6.6 Storage/stability: Section as in section 2.2.6

3. Results and Discussions
3.1 Production and optimization of lipase
The least explored and highly probable source of lipolytic enzyme i.e. resins of *Pinus roxburghii* have been selected for isolation of hyper lipase producing bacteria i.e. G7 on tributyrin agar medium at 37˚C for 24h (Plate 1). Qualitative screening of hyper lipase producing bacteria viz. G7 showed 18.90 mm zone of hydrolysis on tributyrin agar medium and 430 IU/ml lipase activity after quantitative screening using titrimetric method.

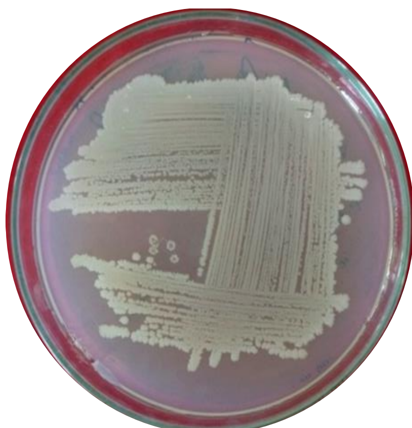

Plate 1: Morphology of hyper lipase producing bacteria i.e. G7

The 16S rRNA gene sequences of the selected isolate viz. G7 has been deposited to National Centre for Biotechnology Information (NCBI) gene bank using Bankit program and has been registered in the databases vide accession number *Paenibacillus polymyxa* G7| MF446912|.
An enhanced enzyme titers of 1740 IU/ml were observed from *P. polymyxa* G7 through OVAT and RSM approach with an increase of 304.65 %, addition of tragacanth gum in place of tributyrin, 4 days incubation period, at 40 ˚C temperature and10.0 pH @ 10.0 % inoculum with 0.20% galactose carbonconcentration.

3.2 Purification and characterization of lipase
Enzyme in purified form is a prerequisite in its studies of structure-function relationships and biochemical properties. Thus to concentrate enzyme and to make it pure in order to increase its efficacy, sequential purification of extracted protein in three steps has been done. Ammonium sulphate precipitation was firstly done to concentrate the protein of interest from the bulk of proteins present in the supernatant. *P. polymyxa* G7 lipase exhibited an increase from 430 IU to 2140 IU after ammonium sulphate precipitation with a purification fold of 1.90 and 99.53% recovery (Table 7). Concentrated lipase was then dialyzed which further concentrated the protein by forcing out the salt and other unwanted proteins from the membrane. *P. polymyxa* G7 rose to 2290 IU with 1.98 fold purification and 5.33% recovery. Gel exclusion chromatography was then performed to further purify the enzyme. Enzyme showed an increased respective enzyme activity of 2510 IU with 2.01 fold purification and 5.84 % recovery. Molecular weight of *P. polymyxa* G7 was found to be 66kDa after SDS.
Purified lipase from *P. polymyxa* G7 was characterized with respect to various physical and chemical characteristics. *P. polymyxa* G7 lipase had expressed an optimal pH 8.0 proving a slightly alkaline nature with maximum relative activity i.e. 101.10%. Behaviour of purified lipase from *P. polymyxa* G7 at varying temperatures ranging from 35-121 °C was explored. Enzyme activity showed a slight increase from 30-35 ˚C. Highest enzyme activity was observed at 35 °C respectively. And upto 90 ºC considerable activity was retained. The enzyme was even active at 121 ºC with 37% relative activity.
Half-life of purified lipase at a temperature of 30-45 °C was 120 min while it was 60 min at 60°C. Enzyme activity reduced to its half in just 30 min at higher temperatures of 70°C. Highest stability was evaluated in press cake of *Brassica nigra* (0.14%) having 1236.84 specific activity and 115.80% relative activity. Shelf stability of the enzyme was studied at 4 °C for 30 days. Lipase from *P. polymyxa* G7 was found to be quite stable with a relative activity of 97.13 and 97.50% after 30 days

3.3 Application of lipase in formulation of functional breads
3.3.1 Physico-chemical characteristics of functional bread
Data in tables 1, 2, 3, 4 and 5 showed that addition of different levels of lipase enzyme of *P. polymyxa* G7 improved different physico-chemical characteristics of all types of functional bread. Addition of enzymes increased specific volume of bread loaves measured in terms of thickness ranging from 6.0 to 6.4 in wheat (W) bread (Table 1 and plate 2), 2.3 to 3.5 in wheat + rye (W+R) bread (Table 2 and plate 2), 2.6 to 3.9 in wheat + amaranth (W+A) bread (Table 3 and plate 2), 3.1 to 3.7 in wheat + buckwheat (W+B) bread (Table 4 and plate 2) and 3.5 to 4.2 in wheat + maize (W+M) bread (Table 5 and plate 2). Vemulapalli *et al.* (1998) [13] had reported that lipase improved the loaf volume of bread during 45, 70 and 90 min of fermentation time.

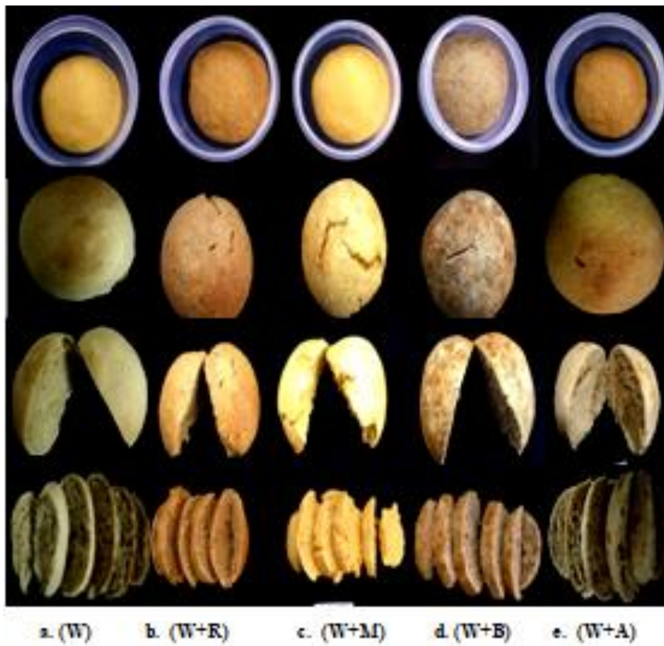

Plate 2: Optimization of lipase of *P. polymyxa* G7 for formulation of multigrain functional bread (a-e)

Table 1: Evaluation of physico-chemical characteristics during the formulation of functional Wheat (W) bread

Sets	Baking weight loss (%)	Diameter (cm)	Thickness (cm)
Control (Without Yeast)	3.77	6	5.8
Control (With Yeast)	10.90	6.5	6
Set A*	12.72	7.0	6.2
Set B**	16.36	7.5	6.4
Set C***	14.54	7.2	6.2
$SE_{(m)}$	0.05	0.02	0.03
$CD_{0.05}$	0.16	0.07	0.08

*Set A: 10ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast

Table 2: Evaluation of physico-chemical characteristics during the formulation of functional Wheat + Rye (W+R) bread

Sets	Baking weight loss (%)	Diameter (cm)	Thickness (cm)/ Loaf height
Control	4.0	5.8	2.3
Control + Yeast	3.84	5.9	2.5
Control + Indigenousinoculum (I)	7.40	6.1	2.8
Set A*	3.84	6.0	2.7
Set B**	9.62	5.9	2.8
Set C***	9.61	6.4	2.9
Set D****	10.71	7.3	3.5
$SE_{(m)}$	0.02	0.03	0.06
$CD_{0.05}$	0.07	0.09	0.18

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 30ppm *P. polymyxa* G7 purified lipase+ Indigenous inoculum (I)

Table 3: Evaluation of physico-chemical characteristics during the formulation of functional Wheat + Amaranth (W+A) bread

Sets	Baking weight loss (%)	Diameter (cm)	Thickness (cm)/ Loaf height
Control	4.44	6.3	2.4
Control + Yeast	6.38	6	2.6
Control + I	10.20	6.2	3.1
Set A*	17.64	6.5	2.5
Set B**	23.07	6.6	3.1
Set C***	28.84	6.7	3.2
Set D****	22.64	8.2	3.9
$SE_{(m)}$	0.06	0.01	0.03
$CD_{0.05}$	0.24	0.05	0.09

*Set A: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 50ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 50ppm *P. polymyxa* G7 purified lipase+ I

Table 4: Evaluation of physico-chemical characteristics during the formulation of functional Wheat + Buckwheat (W+B) bread

Sets	Baking weight loss (%)	Diameter (cm)	Thickness (cm)/ Loaf height
Control	4.08	5.5	2.5
Control + Yeast	4	6.2	3.1
Control + I	5.76	6.1	3.5
Set A*	9.80	6.2	2.8
Set B**	9.80	6.3	2.4
Set C***	15.38	6.7	2.8
Set D****	16.66	7.0	3.7
$SE_{(m)}$	0.03	0.05	0.03
$CD_{0.05}$	0.15	0.18	0.08

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 40ppm *P. polymyxa* G7 purified lipase+ I

Table 5: Evaluation of physico-chemical characteristics during the formulation of functional Wheat + Maize (W+M) bread

Sets	Baking weight loss (%)	Diameter (cm)	Thickness (cm)/ Loaf height
Control	3.92	5.6	3.3
Control + Yeast	3.84	5.9	3.5
Control + I	7.40	6.6	4.0
Set A*	5.66	6.0	3.5
Set B**	11.11	6.4	2.3
Set C***	14.54	7.0	2.8
Set D****	8.77	7.2	4.2
$SE_{(m)}$	0.05	0.03	0.04
$CD_{0.05}$	0.14	0.08	0.13

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 30ppm *P. polymyxa* G7 purified lipase+ I

Similarly effect of baking weight loss i.e. the difference in initial weight of dough and final weight of baked dough is revealed in Table 1, 2, 3, 4 and 5 and Plate 2.The maximum weight loss had been measured i.e. 16.36% (W) over 10.9% (Control), 14.54 % (W + M) over 3.84 % (Control), 16.66%

(W + B) over 4.0%, 28.84 % (W+ A) over 6.38% and 10.71 % (W +R) over 3.84%. The significant weight loss has been noticed in the sets which are adjudged the best with optimized respective doses of lipase which is probably due to the modified structure of wheat and other pseudocereals added to it resulting in entrapment of more quantity of carbon dioxide during fermentation which finally got escaped during baking. Also the major changes during baking *viz*. dough expansion, volume expansion of carbon dioxide and vaporization of water and ethanol had contributed for weight loss. El-Rashidy *et al*. (2015) [14] showed that the addition of lipase enzyme (20ppm) increase specific volume of pan bread loaves from 5.9 in control 7.38 cm.

3.3.2 Compositional analysis of functional bread

Table 6, 7, 8, 9 and 10 depicted functional value of different bread types with highest concentration of proteins from 13.6, 15.6, 16.4 and 10.9 % while PUFA (oleic acid) measured was highest i.e. 0.71, 2.6, 3.9, 2.7 and 2.8 in W, W + R, W + A, W + Band W + M respectively besides being enriched in carbohydrates fraction etc. Spendler *et al*. (2001) [15]; Sirbu and Paslaru, (2005) [16] demonstrated that lipases hydrolyse the ester bond between glycerides and fatty acids producing free fatty acids and more polar lipids in the dough.

Table 6: Compositional analysis of wheat (W) bread

Sets	Free fatty acids (Oleic acid) (grams)
Control (Without Yeast)	0.35
Control (With Yeast)	0.36
Set A*	0.52
Set B**	0.71
Set C***	0.57
$SE_{(m)}$	0.03
$CD_{0.05}$	0.09

*Set A: 10ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast

Table 7: Compositional analysis of Wheat + Rye (W+R) bread

Sets	Carbohydrates	Protein	Free fatty acids (Oleic acid) (g)	Crude fat (%)
Control	49	12.8	0.8	1.9
Control + Yeast	44	12.9	1.3	1.6
Control + I	38	12.2	1.4	1.4
Set A	42	12.2	1.8	1.4
Set B	40	12.9	2.4	1.2
Set C	45	13.3	1.9	0.9
Set D	41	13.6	2.6	0.6
$SE_{(m)}$	0.02	0.05	0.06	0.03
$CD_{0.05}$	0.07	0.13	0.15	0.09

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 30ppm *P. polymyxa* G7 purified lipase+ Indigenous inoculum (I)

Table 8: Compositional analysis of Wheat + Amaranth (W+A) bread

Sets	Carbohydrates	Protein	Free fatty acids Oleic acid (g)	Crude fat (%)
Control	57	13.5	2.6	6.5
Control + Yeast	53	13.8	2.8	6.4
Control + I	55	14.7	2.9	6.1
Set A	58	14.9	3.1	6.2
Set B	61	15.1	3.6	6.0
Set C	62	15.3	3.8	5.6
Set D	58	15.6	3.9	5.2
$SE_{(m)}$	0.04	0.02	0.08	0.07
$CD_{0.05}$	0.08	0.04	0.24	0.18

*Set A: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 50ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 50ppm *P. polymyxa* G7 purified lipase+ I

Table 9: Compositional analysis of Wheat + Buckwheat (W+B) bread

Sets	Carbohydrates	Protein	Free fatty acid (Oleic acid)(g)	Crude fat (%)
Control	59	13.0	0.9	2.8
Control + Yeast	53	13.4	1.1	2.6
Control + I	49	13.9	1.3	2.6
Set A	49	14.6	1.7	2.4
Set B	52	15.2	1.9	2.1
Set C	52	15.8	2.5	1.9
Set D	48	16.4	2.7	1.6
$SE_{(m)}$	0.02	0.03	0.05	0.06
$CD_{0.05}$	0.06	0.07	0.15	0.12

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 40ppm *P. polymyxa* G7 purified lipase+ I

Table 10: Compositional analysis of Wheat + Maize (W+M) bread

Sets	Carbohydrates	Protein	Free fatty acids (Oleic acid)(g)	Crude fats in 100 g (%)
Control	55	7.7	1.2	2.3
Control + Yeast	52	8.1	1.4	2.2
Control + I	50	8.3	1.6	2.0
Set A	51	9.6	1.8	1.8
Set B	52	9.9	1.9	1.6
Set C	55	10.7	2.4	1.3
Set D	54	10.9	2.8	1.2
$SE_{(m)}$	0.04	0.02	0.08	0.06
$CD_{0.05}$	0.07	0.04	0.18	0.13

*Set A: 20ppm *P. polymyxa* G7 purified lipase+ commercial yeast
**Set B: 30ppm *P. polymyxa* G7 purified lipase+ commercial yeast
***Set C: 40ppm *P. polymyxa* G7 purified lipase+ commercial yeast
****Set D: 30ppm *P. polymyxa* G7 purified lipase+ I

3.3.3 Sensorial analysis of baked products

The results in Fig 1, 2, 3, 4 and 5 showed the change in taste,

aroma, texture, softness, color, mouthfeel and overall acceptability for the product. These results clearly prove that all characteristics were affected by the addition of lipase in the final fermented product. Data in same figures exhibited that, the best levels of enzymes for producing bread were different which varied with ingredients. El-Rashidy *et al*. (2015) [14] reported that, the changes in crust color, texture, taste, flavour, appearance and overall acceptability for stored pan bread for 5 days. Lipase was primarily used to enhance the flavour content of bakery products by liberating short chain-fatty acids through esterification. Along with flavour enhancement, it also prolonged the shelf life of most of the bakery products.

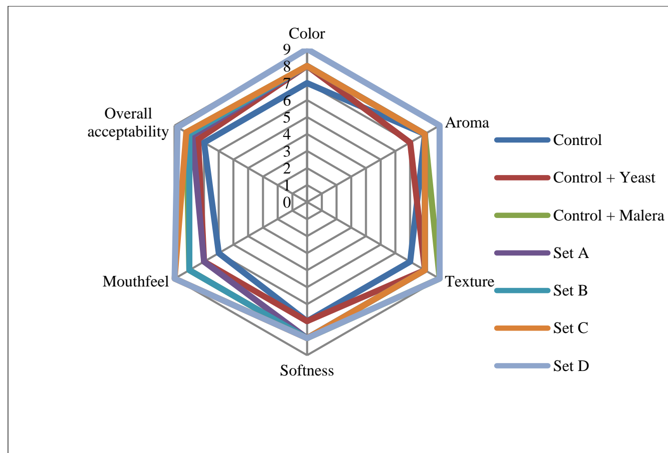

Fig 1: Sensorial evaluation of functional wheat bread

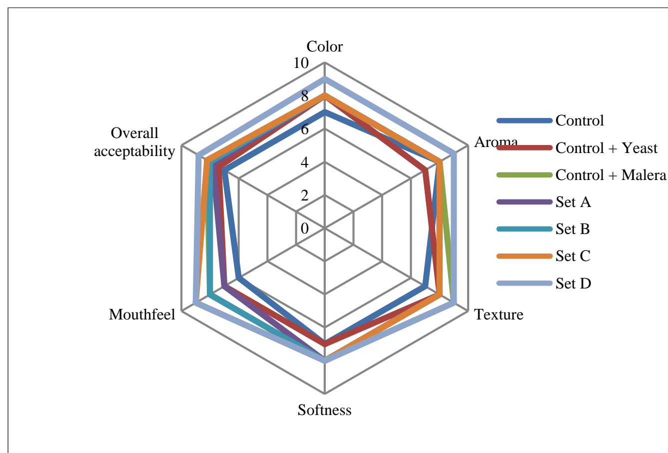

Fig 2: Sensorial evaluation of functional wheat + Rye bread

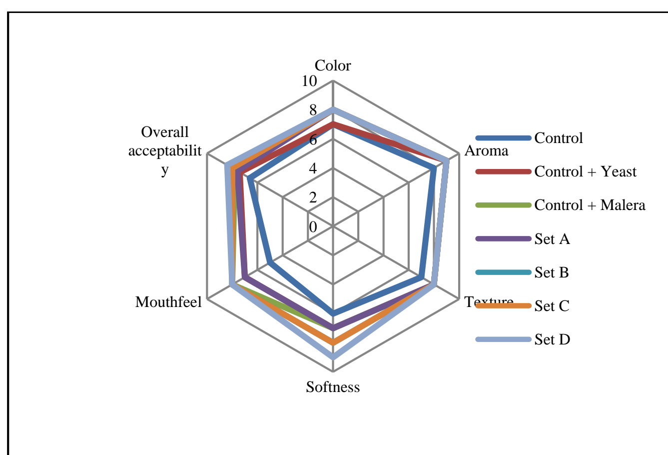

Fig 3: Sensorial evaluation of functional wheat + Amaranth bread

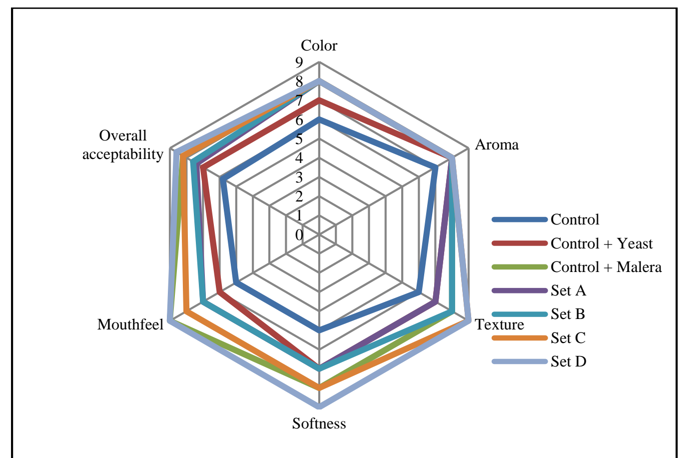

Fig 4: Sensorial evaluation of functional wheat + Buckwheat bread

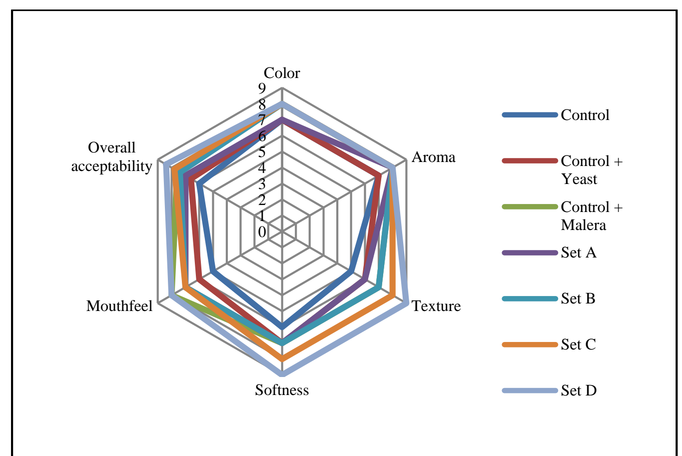

Fig 5: Sensorial evaluation of functional wheat + Maize bread

3.3.4 Storage stability of baked products

The baked breads were kept for 15 days followed by 30 days to explore the different kinds of changes induced in these products (Plate 2). One of very interesting finding noticed was a strong inhibitory effect against growth of moulds on lipase added bread. The fermented products with lipase were highly stable over control (without lipase) proving an outstanding role of lipase of *P. polymyxa* G7 against microbial spoilage of bread. Literature survey revealed that all the characteristics were affected by the addition of lipase enzyme during storage period and 20ppm of enzyme used in preparing bread showed good quality as well as long shelf life (El-Rashidy *et al*., 2015) [14].

Bread is a staple food that is closely related to our daily life. Bread is popular around the world and one of the oldest foods. It is prepared by baking dough which consists of flour, yeast/ lactic acid bacteria as leavening agents and water. Thus healthy baking of bread includes mainly a focus on their formulation ingredients and type of microorganisms used for fermentation. Fermenting microorganisms after reacting with these ingredients impart a specific texture, colour and flavour, baked good volume and bulk density to the final product.

In the present study a successful attempt has been made to formulate a variety of functional breads that combine unique taste and nutritional balance. The strategic approach for the production of light and healthy bread by replacing harmful chemical improves with optimized dose of lipase of *P. polymyxa* G7 in order to enhance product quality and earn a natural and clean label status to bread that is highly sought after by health conscious consumers of modern age. In addition pseudocereal grains like buckwheat, amaranth and

rye are used as bread ingredients which contain high range of beneficial compounds for health. Compounds like flavonoids, phenolic acids and vitamins are available in these grains. Therefore incorporation of these grains in bread dough formulation enhances significantly the nutritional quality of baked product.

Wheat endogenous flour lipids make upto 2.5% of flour on weight basis. Despite of their low levels, wheat flour lipids play a major role during bread making. As shown in the corresponding Plates, the formulations that contained optimized dose of lipase exhibited the highest baking yield value. Increase or decrease in lipase quantity had resulted in a reduced baking volume although variations amongst the lipase using combinations were slight as compared to a significant difference with control where no enzyme was added. This proves the role of lipases in solubilizing the complex lipids like phospholipids, glycolipids etc. present in dough to improve the baking yield of bread. Improvement in dough strength and extensibility is related to lipase enzyme which acts as an activator of oxidative reactions by furnishing substrate namely PUFA's released by lipolysis. Lipase of *P. polymyxa* G7 used in the present study has the special advantage of mainly producing PUFA's (oleic acid).

4. Conclusion

In the present study a successful attempt has been made to formulate a variety of functional breads that combine unique taste and nutritional balance. The strategic approach for the production of light and healthy bread by replacing harmful chemical improves with respective optimized doses of lipase of *P. polymyxa* G7 in order to enhance product quality and earn a natural and clean label status to a variety of breads which are highly sought after by health conscious consumers of modern age. In addition pseudocereal grains like buckwheat, amaranth and rye are used as bread ingredients which contain high range of beneficial compounds for health. Compounds like flavonoids, phenolic acids and vitamins are available in these grains. Therefore incorporation of these grains in bread dough formulation enhances significantly the nutritional quality of baked product.

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