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Improvement of bread Quality by Inclusion of Alpha Amylase from Bacillus Licheniformis

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Abstract: **Amylases have discovered applications in various industries including juice preparing, processing of starch, bread improvement and other maturation forms including malting grain and in baking ventures. In this investigation, amylase extracted from *Bacillus licheniformis* MTCC 1483 was applied for the preparation of buns and for improvement of bread quality. It was evaluated that maximum loaf volume was obtained by an application of 3.5 unit/Kg flour of amylase at 37°C, pH 7.0 for 30min. Tactile attributes of bread *viz.* color, taste, flavor and acceptability were additionally upgraded. Thus, the bread prepared by using alpha amylase showed an increase in loaf volume and bread height with better taste and flavor. Overall results showed applicability of alpha amylase in baking industry.**

Keywords **-** Alpha amylase; *Bacillus licheniformis*; baking, bread quality; rheological properties

I. INTRODUCTION

Industrialization of biotechnological forms has prompted the fast utilization of enzymes in different industries. Amylases are omnipresent and holding most extreme piece of the overall industry of catalysts deals [1]. Amylases are reported from a number of microorganisms such as *Bacillus subtilis* [2], *Penicillium sp.* [3], *Bacillus licheniformis* [4]. Properties such as thermo-stability capacity to hydrolyze native starch, stability at high salt concentration and pH stability at wide range attracts particular attention [5, 6]. Importance of the enzyme increases as per consumer demand for more natural product free from the chemicals additives.

α-amylase is the most commonly used enzyme in baking industry. The addition of enzyme in the dough for the bread increases the bread volume resulting in increased softness of bread for longer duration during storage in comparison to the bread without enzyme [7]. Application of the α-amylase in the bakery processing improves the rheological properties of bread [8]. In addition, small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions that contribute to the development of the browning and attractive baked flavor of the crust [9].

Amylases are currently in use in baking industry as bread improver 1100. But the long exposures of amylase-enriched flour to bakers develop risk of dermatitis or asthma. Therefore, industry is in continuous need of having a safe and effective amylase which can be utilized as bread improver.

Previously, we have reported an α-amylase from *Bacillus licheniformis* MTCC 1483 which had been purified and characterized in detail [10]. Present study was carried out to investigate the application of alpha amylase from *B. licheniformis* MTCC 1483 for bread quality improvement. A comparison was also made with commercial amylase E1100.

II. Materials and Methods

Microorganism and Growth Conditions

The bacterial strain *Bacillus licheniformis* MTTC 1483 was used in the present study [11]. The culture was revived on Brain Heart Infusion broth and maintained by continuous sub-culturing periodically. *Bacillus licheniformis* MTCC 1483 was examined microscopically and Gram's staining was done. The presence of starch was confirmed by pouring iodine solution onto the agar plate having colonies of *Bacillus licheniformis* MTCC 1483.

Chemicals and Flour

All reagents were from Hi- media and of analytical grade. Flour and Paddy straw was purchased from the local market of Mohali, Punjab, India and stored at cool dry place.

Production, Extraction and purification of amylase

Amylase was produced in solid state culture condition using paddy straw as substrate [4]. Substrate was cleaned in running water three times followed by treatment with 1% NaOH for 30 min and drying in oven at 80ºC. The dry substrates were ground in the grinder and sieved through a mesh to obtain equal size particles. Fermentation was carried out by using 5g substrate consisting of 1.81% starch with 70% initial moisture content and 0.94% Tween-80 inoculated with 10% inoculum. Incubation was carried out at 37°C for 48h.

Amylase was extracted as described by Kaur et al. [4]. The crude enzyme was purified by fractionation with ammonium sulphate followed by dialysis and ion exchange chromatography [10].

Amylase assay

Amylase was assayed using supernatant containing crude enzyme by dinitrosalicylic method [12] starch as substrate at pH 7.0, 50°C for 30 min and optical density was taken at 540nm, on UV- spectrophotometer. One unit of enzyme

activity was defined as micromoles of glucose released per minute per one gram of substrate.

Flour characterization
Flour sample was analyzed as per the standard methods of International Association for Cereal Science and Technology (ICC) [13].

Moisture content [14]
Moisture content of wheat flour was calculated by oven heating method. 1g of sample was taken in a procelain dish and kept in an oven at 105°C for 1h. Due to evaporation of water, the weight of the flour decreases. Moisture content was calculated as per following equation 1:

$$Moisture\ content\ (\%) = \frac{Initial\ weight - final\ weight}{weight\ of\ the\ flour\ taken} \times 100 \quad \ldots \quad 1$$

Falling number [15]
To calculate falling Number, 250g of flour was weighed and placed in a viscometer tube. The moisture content of 14% was taken as constant. 25ml of distilled water was poured in tube using a pipette. Tube was shaken thoroughly to form a homogenous mixture. Then the tube was inserted at 100°C. The tube was then placed in falling number instrument. The time taken by the stirring rod to descend a certain distance was noted by the instrument.

Damage Starch

The level of damaged starch is determined by treating the flour starch with amylase. 1g of flour was taken and hydrolyzed with 1U of commercial amylase for 10 min followed by hydrolysis with 1U of amyloglucosidase. The amount of glucose released was measured by dinitrosalycylic acid method [12].

Total protein
Protein content of flour was determined by Kjeldahl method [16]. The nitrogen content of the sample was calculated and multiplied by 5.7, the typical factor used for wheat and wheat flour. Protein content was calculated on 14% constant moisture basis.

Ash content
Ash content was determined by using the muffle furnace method [17]. 3g flour was taken in a crucible dish and burned overnight at 585°C. The samples were cooled in a desiccator and the residue (ash) is weighed. Ash content is calculated as per equation 2 on 14% constant moisture basis.

$$Ash\ content\ (\%) = \frac{Weight\ of\ residue\ (g)}{Initial\ weight\ of\ sample\ (g)} \times 100 \quad \ldots \quad .2$$

Sedimentation value
The sedimentation value of flour was determined by taking 14% moisture as constant. 100 mesh sieve was used to sieve 10g flour. 4g of this sieved flour was then placed in a graduated cylinder to which 50 ml of distilled water was added. The mixture was shaken vigorously by thorough mixing to form a homogenous mixture. To this, lactic acid (85%) and iso-propanol solution (1:1) was added. Methylene blue was used as indicator solution. The solution was again mixed gently. The solution was allowed to stand immediately after mixing and time was noted for 5 min. The sedimentation volume is the volume of slurry in ml after 5 min.

Gluten content and gluten hydration [17]
The weight of gluten is calculated by following equation:

$$Gluten\ content\ (g) = weight\ of\ flour\ taken \times total\ protein\ content\ of\ flour \times 0.8 \quad \ldots \quad 3$$

Gluten absorbs water two times to its own weight, therefore, the water absorption by gluten is calculated as

$$water\ absorbed\ by\ gluten\ (g) = weight\ of\ gluten\ (g) \times 2 \quad \ldots \quad 4$$

$$Water\ (\%) = \frac{water\ absorbed\ by\ gluten}{62} \times 100 \quad \ldots \quad 5$$

Dough preparation and baking
Sieved 500 g of wheat flour and mixed with 1% sucrose, 4% yeast, 0.5% NaCl, and 0.5% vegetable oil in equal quantity of warm distilled water. The batter is mixed with flour from the dough. Flying fermented by mixing one-fifth of the crushed yeast and all the sugar in one-third of the tepid water (37°C). The mixture was allowed to stand for 5-10 minutes until it was broken and floated on the surface. A small amount of refined wheat flour (5 g) is added to the suspension, beating manually until a thick paste is formed. The paste was kept at 37°C for 10 minutes. The rest of the ingredients and water along with the paste are mixed for 5 minutes and the dough is made with 0.5 g of ghee. The fermentation was done in a clean, clean glass beaker at 30 ° C and 75% relative humidity for 30 minutes. After 20 minutes of fermentation, the batter was pulled back and allowed to continue for an additional 10 minutes. The fermented dough is evenly divided into two parts, rounded, molded and placed in two oily pans (12 × 9 × 8 cm). Proofing was carried out at 35°C for 45 minutes at 80-85% relative humidity. Baking was done at 250°C for 15 minutes. After baking, the bread loaves were allowed to cool at 1 for 28. C. Slicing and packaging were done after the bread volume and weight were determined.

Determination of pH of dough preparation
The pH of the dough was determined with the help of pH meter.

Optimization of baking conditions by amylase with OVAT method
The conditions for bread baking were optimized with respect to the parameters *viz.*, enzyme dose, reaction time and incubation temperature by varying one factor at a time keeping the other constant. Conditions optimized in previous experiment were used in subsequent experiments.

Enzyme dose
To optimize the enzyme dose for bread making, dough was kneaded with amylase varying in the range of 0-5 IU/kg of flour. Dough was prepared as per method described above. In control, dough was made under the same conditions expect enzyme which was replaced with equal amount of water. Baking was done as described above. The specific volume for all the baked breads was measured immediately

by the rape seed method. A positive control consisting of commercial amylase and a negative control with no amylase was taken as control.

Temperature

To optimize the temperature of enzyme reaction, dough was kneaded by adding the optimized dose of enzyme to the flour. Dough was prepared as per method described above. The temperature of enzyme reaction was varied from 30-42ºC. Fermentation was carried out for a time period of 45 min. A positive control consisting of commercial amylase and a negative control with no amylase was taken as control. In negative control, dough was made under the same conditions expect enzyme which was replaced with equal amount of water. Separate controls were made for each time to rule out the effect of temperature on fermentation. Baking was done as described above. The specific volume for all the baked breads was measured immediately by the rape seed method.

pH

Buffers were used in place of distilled water to study the effect of pH on amylase dependent bread leavening. pH was varied from 5.0-7.5 using 50mM buffers (acetate buffer 5.0-5.5; phosphate buffer 6.0-7.5). Spongy dough was prepared and put into trays and allowed for proofing in the proofing chamber for 20 minutes. Baking was done as described above. Bread thus prepare, was cooled to room temperature for 30 minutes. A positive control consisting of commercial amylase and a negative control with no amylase was taken as control.

Incubation time

To optimize the incubation time for bread making, dough was kneaded by adding the optimized dose of enzyme to the flour. Dough was prepared as per method described above. Fermentation was carried out for a time period of 15-60 min. A positive control consisting of commercial amylase and a negative control with no amylase was taken as control. In negative control, dough was made under the same conditions expect enzyme which was replaced with equal amount of water. Separate controls were made for each time to rule out the effect of fermentation time. Baking was done as described above. The specific volume for all the baked breads was measured immediately by the rape seed method.

Bread evaluation

The outcome of enzyme applocation on the superiority of bread was determined by using following parameters:

Loaf volume

Bread size was measured by rapeseed displacement method [13]. Fixed dimension box (6.00 cm x 15.00 cm) of internal volume 1674 cm^3 was placed in a tray and filled with rapeseed. The seeds were transferred from the box to the container and weighed. One weighed bread was placed in the box and the weighted seeds (500 g) were used to fill the box and rebuilt as before. The weight of the seeds displaced by the bread and the weight of the seeds around the loaf are calculated from the weight of the overspill. Analysis has been done in triplets [18].

Sensory evaluation

Samples of the coded bun were tested by 10 panelists by the hedonic scale at room temperature (25°C) [19]. Color, taste, taste, softness and overall acceptability are the evaluated characteristics.

Texture analysis

Texture profile analysis of bread samples were carried out at Punjab Biotechnology Incubator, Mohali. Rheolological properties like hardness, cohesiveness, springiness, gumminess and chewiness were studied.

Statistical analysis

All the experiments were carried out in triplicates. The data obtained were subjected to analysis of variance methods.

III. Results and discussion

Baker's yeast *Saccharomyces cerevisiae* utilized fermentable sugar to convert it into CO_2 and ethanol. The amount of CO_2 released depends upon the amount of fermentable sugar. Wheat flour, the major ingredient of bread, contains approximately 75-80% starch which cannot be directly utilized by yeast. Therefore, to make the starch accessible to yeast, it needs to be digested with enzyme so as to convert it into simplest sugar. Important portions of the sugar consumed during fermentation are obtained by the enzymatic hydrolysis of the starch (DS) and fructan [20]. Yeast invertase, the endogenous amylase in starch, hydrates the damaged starch into maltose, and fructose into glucose [20]. Thus, glucose, fructose and maltose are the main substrates for fermentation as a result of hydrolysis. Nevertheless optimal loaf volume cannot be obtained by indigenous amylase of flour. Therefore, addition of external amylases is the key to bread industry.

In baking industries, commercial amylases are used which are obtained from fungal sources. Prolonged use of these amylases may cause health effect to the bakers. Therefore, requirement of a safe and effective amylase for bread baking is currently in demand. In the present study, α-amylases obtained from *Bacillus licheniformis* MTCC 1483 was used for the study of its effect on the bread formation.

Quality of wheat flour

Wheat flour obtained from market was analyzed for various properties like ash content, moisture level, starch content, total protein, gluten and alcoholic acidity (Table 1).

TABLE: 1 ANALYSIS OF WHEAT FLOUR PROPERTIES

S.No.	Test Conducted	Values Obtained	Standard
1	Moisture content	10.12%	<14%
2	Ash content	0.32%	<1.0%
3	Falling number	268 sec	>200 sec; <350 sec
4	Total Protein	13%	13-14%
5	Starch content	85.13%	>75%
6	Sedimentation value	65ml	45-65ml
7	Gluten	13%	8-14%
8	Gluten hydration	38.58%	

The moisture content of the flour was well in range. Higher moisture content makes the flour more vulnerable to contamination by bacteria and fungi. The protein content of 13% showed that the wheat is of high protein quality having good dough strengthening properties giving good extensibility and elasticity to bread. Ash reflects the inorganic or mineral content of the flour as it is utilized as an indicator of the quality of flour since it indicates how much bran in the flour is present.
For most bread making applications, bakers look for excellent quality flours with high protein levels and highest purity in terms of endosperm content.

Increase in ash content resulted in increased level of non-endosperm content giving poor baking quality. The flour used in present study have 0.32% ash content which is due to the endosperm portion of the kernel, making it good for baking application. The Falling Number (FN) test is used to calculate wheat and flour alpha amylase activity. Falling number of 268 sec of wheat flour showed that internal amylase is not present in high amount and flour is good for baking. Gluten content and its hydration is necessary for baking applications as it helps in activating the wheat gluten protein.

Glutens, in particular Triticeae glutens, have unique vi scoelastic and adhesive properties that include dough elasti city and chewy texture [21-23]. In water, gliadin and glutenin proteins interact to establish bonds and form small protein strands. A sufficeintly hydrated gluten network can make it more elastic, stretchable and extensible. Better sheeting and moulding of dough is resulted with improved cell structure of finished product. Amino acids such as glutamine, threonine and serine are responsible for the water binding capacity of gluten. When hydrated, gliadin is viscous and can be stretched to a thin strand that flows easily with gravity like glue. On the other hand, hydrated glutenin is very elastic and has a large resistance to deformation and stretching forces. When these two proteins are combined, they form a three-dimensional, continuous network called gluten. Subsequently, gluten has both viscous and elastic properties. It is impossible to create dough with optimum handling properties without hydrating gluten proteins first. If the dough is not sufficiently hydrated, wheat flour proteins can't participate in forming a network during baking. In the present study, gluten hydration level was come out to be 38.6%. Therefore, approximately 40% water is required to fully hydrate the wheat flour.

The sedimentation test is a physicochemical test that helps provides information on the wheat baking quality. It depends on the suspension of flour in a dilute alcohol and acid solution which causes the flour particles to sediment. Flours which show higher rates of hydration absorb more water. Sedimentation value is expressed as volume in ml of settled gluten and is dependent on the quantity as well as quality (strength) of the gluten in the flour sample. As the amount of water absorbed increased, the compactness of gluten particles decreases due to reduced density of the gluten particles, therefore it sink or sediment more slowly. The results suggested that the wheat flour taken was highly suitable for baking applications.

Effect of amylase concentration on bread baking

Amylase was mixed in varying concentration (0-5 IU/kg) with the flour while dough preparation. The collected findings are depicted in Fig. 1. It was noticed that 3.5IU/kg amylase resulted in maximum loaf volume of bread. Amylases work in wheat flour on weakened starch and degrade it into dextrins. During the dough fermentation, the yeast gets continuous nutrition from the proofing and the early baking stage and thus work more efficiently.

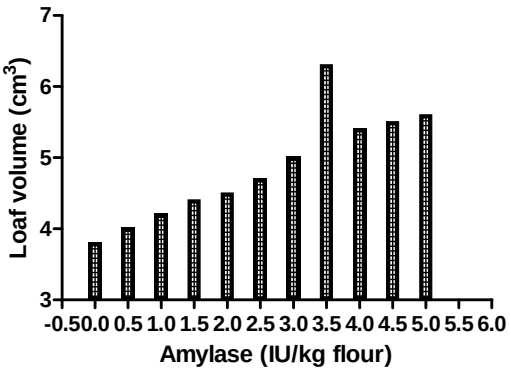


Fig. 1: Effect of amylase treatment on bread loaf volume

This increased the amount of bread and the texture of the c rumbs. Ait Kaki El-Hadef El-Okki et al. [7] have also reported an improvement in bread volume by the *Rhizopus oryzae* FSIS4 amylase treatment.

Effect of pH on bread baking

Buffers of different pH values were used to prepare dough for bread baking. Maximum loaf volume was obtained at pH 7.0 (Fig. 2). The results were in accordance to optimum pH of *B. licheniformis* amylase activity. Shobna et al. [24] also carried out the baking application at optimum pH of Bacillus subtilis activity and described that maximum loaf volume can be achieved at this pH.

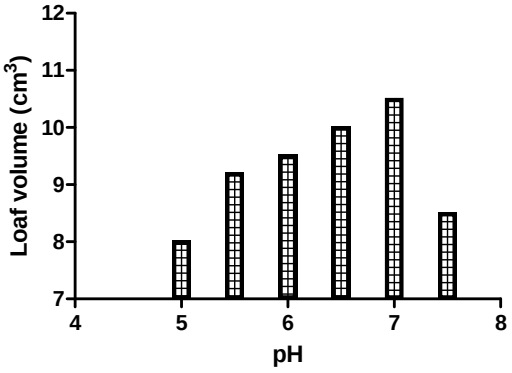
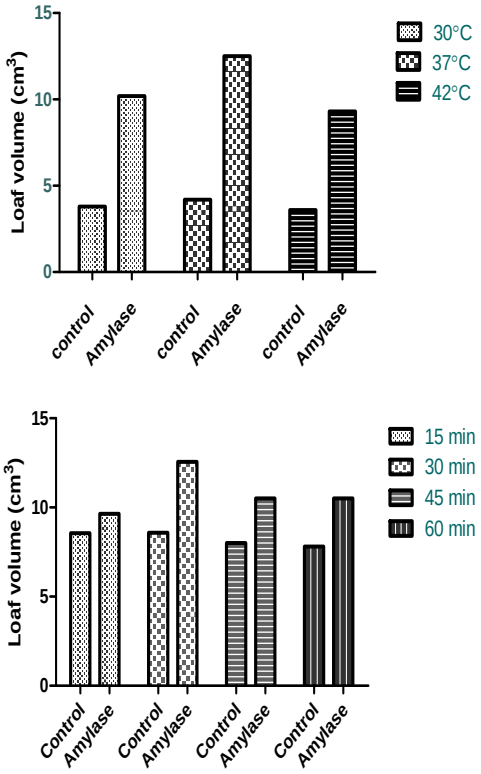


Fig 2: Effect of pH on loaf volume

Effect of temperature and incubation time on bread baking

Effect of temperature on bread baking with amylase was studied at three different temperatures. Maximum loaf volume was obtained at 37°C (Fig. 3a). Treatment of dough with amylase was carried out for different time intervals (Fig. 3b). It was observed that maximum loaf volume was obtained at 30 min of incubation.



(b)

Fig. 3: Effect of various parameters on specific loaf volume of bread (a) temperature (b) incubation time

Sensory characteristics at different enzyme concentration

The sample of sliced bread was served in cleaned white plate to panelist at room temperature (28˚C) for sensory evaluation. Attributes evaluated in bread were color, taste, flavor, and overall acceptability of breads. The results are shown in the Fig. 4. All the five persons nominated for sensory evaluation liked the taste and flavor of amylase containing bread. The bread was softer than the non-amylase bread and is overall acceptable by the consumer.

Textural analysis

The result of the textural analysis of control and amylase containing bread were as listed in Table 2. It was notices that application of *B. licheniformis* amylase lead to better rheological property of bread when compared to control and commercial amylase treated bread. Chewiness, gumminess, coheshiveness and springiness of amylase treated bread decreased resultin in formation of more soft bread having large pores (Fig. 5).

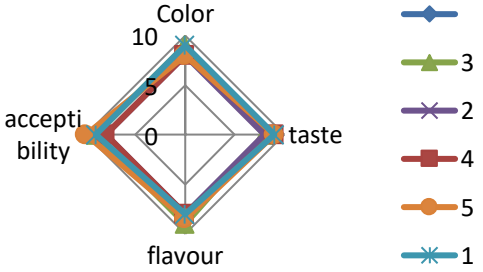


Fig. 4: Sensory evaluation of bread

Fig. 5 Baked bread (a) control (b) amylase treated (c) commercial amylase

TABLE 2: RHEOLOGICAL PROPERTIES OF BREAD

Parameter	Control	Commercial amylase	B. licheniformis amylase
Moisture %	41.18	41.87	42.26
Protein %	8.25	8.26	9.00
Sugar %	3.15	3.05	3.00
Hardness (g)	5140.862	4190.263	3508.561
Adhesiveness (g/sec)	0.625	0.554	0.325
Resilence %	43.125	44.184	46.458
Cohesiveness	0.787	0.728	0.678
Springiness %	100	97.25	92.56
Gumminess	4325	3550	2996
Chewiness	4234	3545	2927

Our result are consistent with other studies where author suggessted that application of alpha- amylase in bread baking contributes to the quality of bread [25]. David et al. [26] also reported that a small dose of β-amylase (10g/100kg) improved the quality of flour for bread making. They suggested that amylase should alter the rheological properties of dough to improve the quality of bread.

IV. CONCLUSION

Application of amylase from *Bacillus licheniformis MTCC 1483* enhanced and improved the quality of bread. The optimum reaction temperature, pH and incubation period were found to be 37°C, 7.0 and 30min which gave maximum specific volume of bread. Production of bread using amylase showed breads having increased volume, increased firmness, improved color and taste of bread. Thus, potential of amylase from *Bacillu slicheniformis MTCC 1483* can be employed in food industry for better bread production without the use of any chemicals.

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