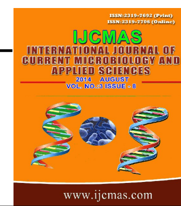

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Original Research Article

Protein degradation in wheat sourdough fermentation with *Lactobacillus plantarum* M616

Jinshui Wang*, Sen Yang, Yanli Yin, Feng Jia and Changfu Zhang

PG College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, P. R. China
**Corresponding author*

Keywords

Sourdough; *Lactobacillus plantarum*; proteins; hydrolysis

A B S T R A C T

Hydrolysis of wheat proteins during sourdough fermentation was determined in the present study. Sourdoughs were characterized with respect to cell counts, pH, TTA and proteolytic activity as well as the quantity of total proteins and water-soluble proteins. Moreover, composition analysis of total proteins and water-soluble proteins using SDS-PAGE was carried out. SYL dough showed a decrease in pH and increase in TTA during fermentation. Sourdough fermentation using *Lactobacillus plantarum* (SYL) resulted in hydrolysis and solubilization of wheat proteins. It demonstrated that protein fractions hydrolysis in sourdough were mainly caused by pH-dependent activation of cereal enzymes according to change in proteolytic activity.

Introduction

Use of the sourdough as a means of leavening agent is one of the oldest biotechnological processes in cereal food production (Rupesh et al., 2011). Sourdough fermentation is a traditional process employed in wheat and rye baking. In the last long time, sourdough fermentations were widely replaced by straight dough processes in industrial production. However, this trend towards a reduced use of sourdough was reversed in the past years. The industrial use of sourdough predominantly primarily aims to improve bread quality, and to replace additives. This shift of the technological aims resulted in the development of novel fermentation technologies and starter cultures with defined metabolic properties (Gobbetti et al., 2007; Brandt, 2007). The use of sourdough in cereal food making influences all aspects of food quality. These technological effects included inhibiting the growth and development of spoilage microorganisms (Lavermicocca et al., 2000; Gänzle et al., 2002), improving the flavor (Thiele et al., 2002) and texture, prolonging shelf-life (Collar et al., 1994; Armero et al., 1998), and increasing the nutritional quality of cereal foods (Liljeberg et al., 1996). These changes in processing and eating quality of cereal food are associated with bioconversion of wheat flour components at the dough stage (Vermeulen et al., 2006; Park et al., 2006; Thiele et al., 2002).

The optimization of the sourdough process for industrial applications in wheat food making requires insight into the biochemical mechanisms responsible for the quality of sourdough fermented foods. Evidence for the impact of specific metabolic activities on cereal food quality was provided, for instance, concerning the generation of flavor precursors and flavor volatiles (De Vuyst et al., 2005). The formation in dough of expolysaccharides by *Lactobacillus sanfranciscensis* improves wheat bread texture (Thieking et al., 2003), but few data are available on protein changes affecting the texture and quality of sourdough fermented wheat foods.

Dough properties and the eating quality of wheat food are strongly influenced by the content, composition and structure of the flour protein. The quality proteins may be improved by the fermentation process. Small peptides and free amino acids released by proteolysis in sourdough fermentation system during fermentation are very important for rapid microbial growth as well as fermentative activity of yeasts and lactate production by LAB and as precursors for flavor development of fermentated wheat foods (Gänzlea et al., 2008).

The proteolytic/peptidolytic activity of LAB can contribute to hydrolysis of bitter peptides and liberation of bioactive peptides (Mugula et al., 2003). However, proteolytic degradation of wheat flour proteins may adversely affect rheological characteristics of wheat doughs and wheat food texture.

The aim of the present study was to investigate the changes in wheat flour proteins occurring during sourdough fermentation. Meanwhile, some factors being responsible for proteolytic activities such as pH, microbial growth, TTA, were also analyzed.

Materials and Methods

Materials

Commercial white wheat flour purchased from local market was used in this study. The used flour contains 12.7 % of protein, 0.43 % of ash and 10.4 % of moisture content. Angel active dyr yeast was provided by ANGEL YEAST Co., Ltd (Yichang, Hubei, China). *Lactobacillus plantarum* M616 was isolated and stored by our laboratory.

Sourdough fermentation and preparation of sample

L. plantarum M616 was growth in MRS broth at 37 °C for 16 h, and then transferred into 75 mL MRS broth (inoculation amount 2 %) for 8 h at 37 °C (cell counts 10^9 cfu/mL). The cell was obtained by centrifugation at 3000×g for 10 min, washed twice by sterile water, and resuspended in sterile water (cell counts 10^9 cfu/mL).

Sourdough was prepared by adding 245.5 g suspended and 5.5 g yeast to 500 g flour (*L. plantarum* M616 of dough 10^8 cfu/g) (SYL). Yeast dough (SY) was prepared by adding 5.5 g yeast to 500 g flour, which had been activated by 245.5g water for10min at 30°C. The control group (CK) was prepared by adding 245.5 g water to 500 g flour. After mixing, the dough was fermented for 24 h at 30 °C in the fermenting box. Samples were taken at different time (0, 2, 4, 6, 8 and 24 h) into refrigerator. All samples were stored after freeze drying and milled into flour.

Microbial counts

1 g of each sample was suspended in 10 mL sterile physiological saline and homogenized in vortex. To counts the number of *L. plantarum* M616 cells, each homogenate

was tenfold diluted serial and spread on MRS agar contained 0.01g/L cycloheximide as a yeast inhibitor (GUL H 2005 and REHMAN S-U 2006). Culture dishes were incubated for 48 h at 37 °C. In order to count the cells of Yeast, each homogenate was spread on YPD medium without inhibitor and incubated for 36 h at 28 °C.

Determination of pH, total titratable acidity (TTA) and dough volume

The pH was determined according to methods of AACC 02-52.01. To measure the pH of the dough, 10 g of the wheat sourdough was placed in a beaker containing 90 mL of distilled water, mixed homogeneously, and then left for 30 min at room temperature. The resultant supernatant was measured with a pH meter. For measuring total titratable acidity, 10 g of the wheat sourdough was placed in a beaker containing 90 mL of distilled water and vigorously stirred for 30 min. After which, 0.5 mL of 1.0% phenolphthalein indicator reagent was added, followed by titration with 0.1 M NaOH, The acidity was measured according to the point at which a pink color was maintained for 30 s. To measure dough volume, 10 g of the dough was placed in a 100-mL measuring cylinder and fermented under primary fermentation conditions, after which the swollen top part of the dough was measured.

Measure of proteolytic activity (free amino nitrogen content)

Proteolytic activity was determined using the ninhydrin test as described by Thiele et al. (2002). This method was employed from other research studies to determine the proteolytic activity in sourdoughs (Thiele et al., 2002). Measurements were performed with a FLUO star Omega at a wavelength of 570 nm, analyzing three independent replicates.

Separation of proteins by SDS-PAGE

Sourdough total protein extracts was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Schagger and Von Jagow (1987). Before the extraction, 125 mg of each sample was dried and milled, then put into a 1.5 mL eppendor tube. In every step of the extraction 1.0 mL of extraction buffer was used and centrifugation was at 1.1×10^{5} g for 10 min. In the first step, the salt soluble protein was extracted by 0.5 M NaCl and 0.05 M Tris-HCl buffer for1.5 h in shaker at 50 °C. In the second step, with centrifugation and two washes, the precipitate was mixed with 1mL 70 % alcohol for 1.5 h in shaker at 55 °C. The fraction presumably contained gliadin. At last, In order to obtain glutenin, the precipitate was incubated with SDS-SB in orbital shaker at 55 °C (0.07 % β-mercaptoethanol was contained as a reducing agent). SDS-SB was prepared by mixing 5mL of 0.5M Tris-HCl (pH6.8), 4mL of glycerol, and 8mL of 10 % SDS, with 3mg bromophenol blue (Loponen et al., 2004). The proteins were analyzed with 10 % and 12 % Tris - HCl SDS-PAGE gels (Laemmli, 1970).

Statistical analyses

Amounts of pH, TTA, FAN, total proteins and water-soluble proteins were determinations four times. Consequently, a variance analysis (ANOVA) was performed on each experiment to determine the effect of fermentation at 95 % or 99 % level.

Results and Discussion

Growth of lactic acid bacteria and yeast during sourdough fermentation

The original cell amounts of *L. plantarum* M616 and yeast in the dough samples in

preparing sourdough were 10^6 CFU/g in this study. No addition of these two microbial was made in preparation of CK. Trace amounts of yeast and *L. plantarum* M616 were detected in original CK dough. There were no obvious changes in cell counts during fermentation. The same phenomenon was found in SY (little *L. plantarum* M616) and SL (little yeast) doughs. During the early time, *L. plantarum* M616 presented as exponential phase after 2 h growth of lag phase in SL dough, while the yeast directly exponentially grew in SY dough. Moreover, as shown in Fig. 1, the reproduction speed of yeast in SY dough was faster than that of SYL dough during fermentation. The cell counts of yeast reached to 10^9 CFU/g at 10 h of fermentation in SY dough. The growth of *Lactobacillus* made the cell counts of lactic acids increased and the pH value decreased in SYL dough. In SYL dough, the growth of yeast was depressed due to *L. plantarum* M616. As a result, the count of yeast in SYL dough reached to 10^8 CFU/g which was obviously much lower than that of SY dough. It showed that *Lactobacillus* strongly inhibited the growth of yeast in sourdough, but in the early time of fermentation, yeast played a leading role in promoting the proofing of dough. After the metabolic acid and flavor substances produced by *Lactobacillus*, the flavor and antibacterial property of dough were increased.

Change in total titratable acidity (TTA) and pH

The titratable acidity and pH are important during sourdough fermentation. According to Fig. 2, pH of CK dough and SY dough changed slightly and almost remained constant and had no obvious change. However, pH value of SYL dough decreased significantly ($p < 0.05$) after of 2 h of the fermentation compared the two samples above mentioned. The lowest pH was found at 10 h of fermentation. To the opposite, amount of TTA of SYL dough increased during the initial stage of 10 h of fermentation. The other samples had no obvious change in TTA. The growth of lactic acid bacteria resulted in the acidification of sourdough during fermentation (Clark et al., 2003).

Change in dough volume during fermentation

In the fermentation phase, the carbon dioxide produced by the yeast is collected in the gas cells formed during mixing, and then resulted in volume increases of dough. This stage is the most important for development and quality of sourdough in process. Its characterization is therefore necessary to control and improve the quality of the final product. Fig. 3 presented the comparison of sourdough volume after 20 h of fermentation. CK sample showed slight increase in dough volume at the end of fermentation (21.5 mL) compared to initial fermentation stage (18.6 mL). However, Significant ($p < 0.05$) increases in dough volumes of SY and SYL samples (27.8 mL and 29.9 mL) at the end of fermentation were found compared with their initial stage (19.2 mL and 19.3 mL).

Effect on proteolytic activity and on free amino acids in wheat sourdoughs

The free amino nitrogen (FAN) content was generally used to measure the proteolytic activity in wheat doughs. FAN was commonly used to monitor the extent of the overall proteolysis upon sourdough fermentations (Loponen et al., 2007; Thiele et al., 2002). An increase of proteolytic activity was observed during all fermentation stage as shown in Fig. 4. These three samples showed a lower FAN content until 5 h of fermentation. A slow increase

upon fermentation time was observed in CK. Higher FAN content was monitored during fermentation in SYL. SYL exhibited the highest content of FAN (52.1± 2.35 $mmol.kg^{-1}$ sourdough) at the end of fermentation and CK showed the lowest content (18.3± 1.26 $mmol.kg^{-1}$ sourdough).

Change in protein of sourdough during fermentation

Total protein content in sourdough

The content of total protein in sourdough began to decrease during fermentation in the three samples as presented in Fig. 5. Slow decrease in total protein content was found during fermentation in CK, and total protein content in CK sourdough kept constant after 6 h of fermentation. The initial wheat flour showed 12.7 ± 0.15 % of protein. The CK contained 9.1 ± 0.13% of total protein at 24 h of fermentation. While, significant decrease of total protein content in SYL was monitored at 24 h of fermentation (5.1 ± 0.08 %) compared with initial fermentation stage (13.1 ± 0.18 %). The decrease in the content of total protein in sourdough was resulted from the degradation of wheat flour proteins due to the action of proteolytic enzymes (proteases) and lactic acid bacteria. Proteases of wheat flours are generally grouped into proteinases and peptidases (Gänzlea et al., 2008; Loponen 2006). Proteinases catalyze protein degradation into smaller peptide fractions; peptidases hydrolyse specific peptide bonds or completely break down peptides to amino acids. The proteolytic activity of wheat flours is attributable mainly to aspartic proteinases and carboxypeptidases and both protease groups are active under acidic conditions (Mikola 1986). Aspartic proteinases of wheat are partly gluten-associated (Bleukx et al., 1998, 2000). Flour protein was hydrolyzed into peptides and free amino acids. Amino acids affected the taste of fermented foods and, in particular, are important precursors for volatile flavor compounds. Lactic acid bacteria in sourdough relyed on the proteolytic system to meet their nutritional requirements with respect to amino acids during fermentation (Kunji et al., 1996). The main components of this system are the cell-envelope-associated serine proteinase, amino acid and peptide transport systems, and a range of intracellular peptidases (Guédon et al., 2001). The initial step in protein degradation is performed by the proteinase and uptake of peptides is the main route of entry of organic nitrogen into the cell (Juillard et al., 1995).

SDS-PAGE pattern of protein fractions in sourdough

To measure the composition of proteins by sourdough fermentation, the proteins from SYL (Fig. 6-A) and SY (Fig. 6-B) dough were analyzed by SDS-PAGE (Fig. 6). SDS-PAGE analysis of protein extracts showed that the hydrolysis of wheat flour proteins was extensive in the SYL (Fig. 6-A). The protein fractions in SYL dough underwent an extensive hydrolysis at the beginning of the fermentation, resulting in the virtual disappearance of some protein bands with higher molecular weight from their respective gels. Some protein bands with lower molecular weight appeared in their gels upon the time of fermentation in SYL dough. It showed that the protein fractions with higher molecular weight were degraded into small peptides and free amino acids in fermentation. Meanwhile, No clear degradation of protein occurred in SY dough during the fermentation (Fig. 6-B).

SDS-PAGE pattern of salt-soluble protein in sourdough

Proteolysis made soluble in salt for the

protein fractions of wheat flour, especially wheat gluten proteins and improved the nutritional value and effective. In order to study the change in salt-soluble protein in sourdough, the salt extract from total protein fractions of sourdough was analyzed using SDS-PAGE (Fig. 7). No obvious change in number of protein bands was found in SY dough during fermentation, however, color intensity of some bands gradually became light upon fermentation time (Fig. 7-B). It indicated that sourdough salt-soluble proteins were limited hydrolyzed in presence of yeast. Significant difference ($p < 0.01$) in SDS-PAGE pattern was found for SYL dough in fermentation (Fig. 7-A). Especially, at 4 h of fermentation, the number protein bands with high and moderate molecular weight began to obvious decrease. Further fermentation (after 6 h) resulted in disappearance of nearly all protein bands. Salt-soluble protein fractions in sourdough were extensively hydrolyzed in the presence of Lactic acid bacteria in the fermentation system. Lactic acid bacteria played an important role in proteolysis or degradation of wheat flour proteins.

The present study delivers some valuable information about the influence of lactic acid bacteria on proteolytic activity and on protein degradation in wheat sourdoughs. Endogenous proteases in wheat flour played a main role during proteolysis in sourdough and this activity depends on fermentation time, pH and growth of lactic acid bacteria. Moreover, the content of total protein in SYL dough decrease upon fermentation time compared with CK dough. Of which, hydrolysis of salt-protein fractions was main reason for decrease in content of total protein. These changes can have an influence on quality of sourdough.

Figure.1 Growth curve of lactic acid bacteria and yeast during sourdough fermentation. CK-Y, yeast in CK dough; CK-L, lactic acid bacteria in CK dough; SY-Y, yeast in SY dough; SY-L, lactic acid bacteria in SY dough; SL-L, lactic acid bacteria in SL dough; SL-Y, yeast in SL dough; SYL-L, lactic acid bacteria in SYL dough; SYL-Y, yeast in SYL dough.

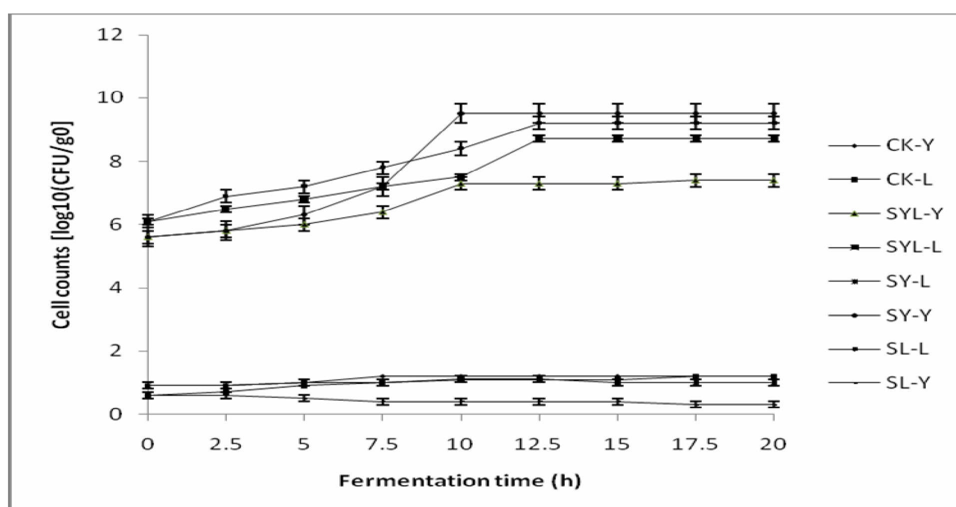


Figure.2 Variations of pH and TTA in sourdough fermentation. ▼, pH of SYL dough; ●, TTA of SYL dough; ▲, pH of SY; ★, TTA of SY dough; ■, pH of CK; ◆, TTA of CK dough.

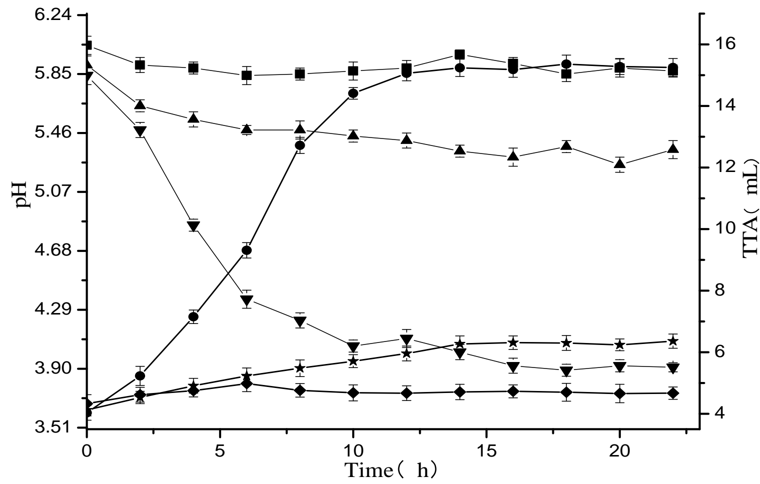


Figure.3 Change in dough volume during fermentation

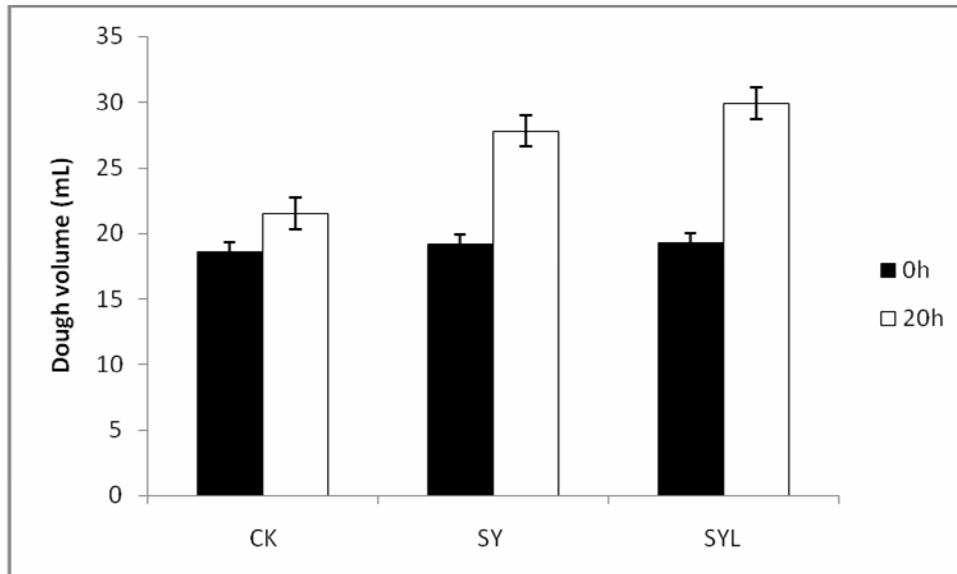


Figure.4 Development curve of free amino nitrogen during fermentation without microbial (CK), with yeast (SY) and with yeast and *L. plantarum* (SYL)

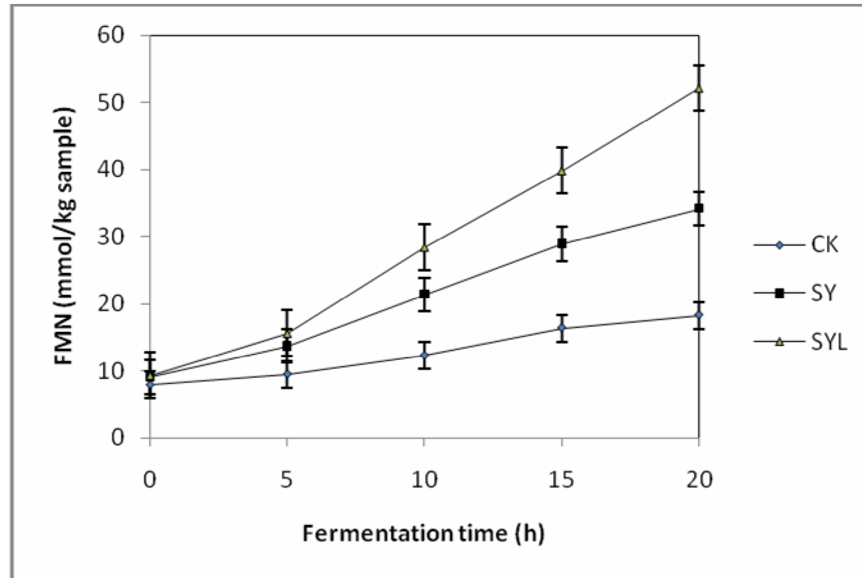


Figure.5 Change profile of total protein content in sourdough during fermentation without microbial (CK), with yeast (SY) and with yeast and *L. plantarum*.

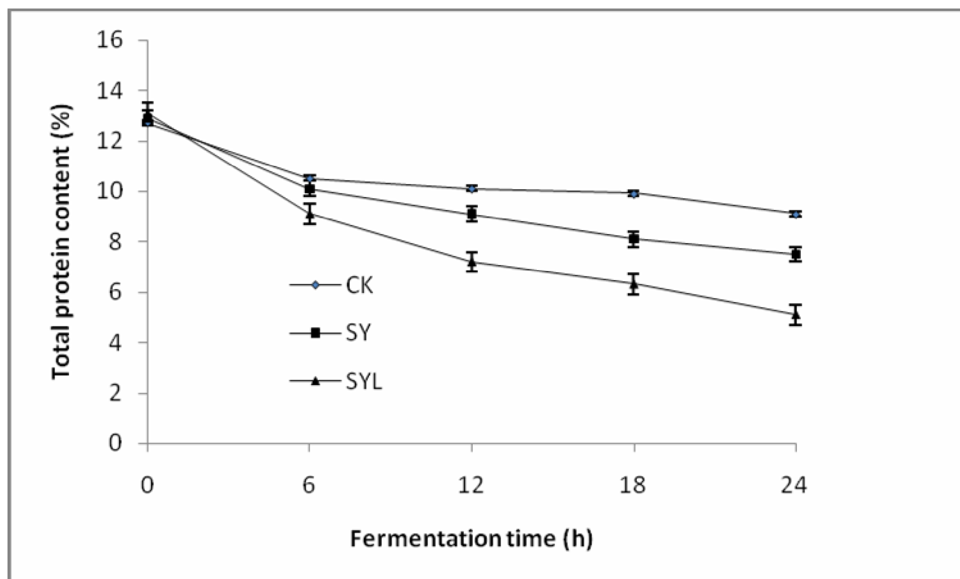


Figure.6 SDS-PAGE pattern of protein in sourdough during fermentation. A- SYL dough, B- SY dough

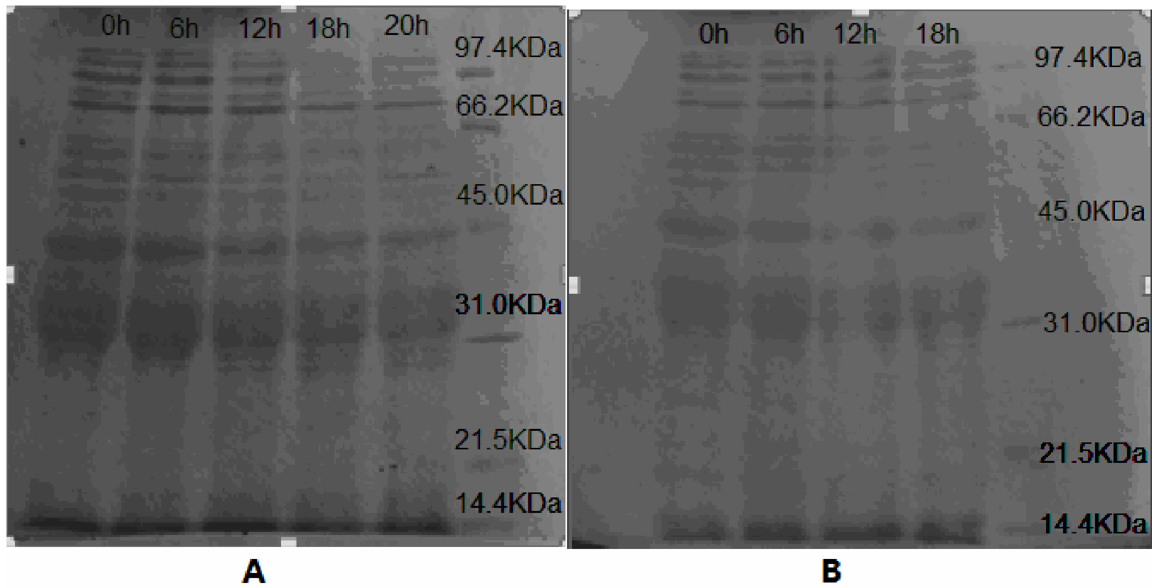
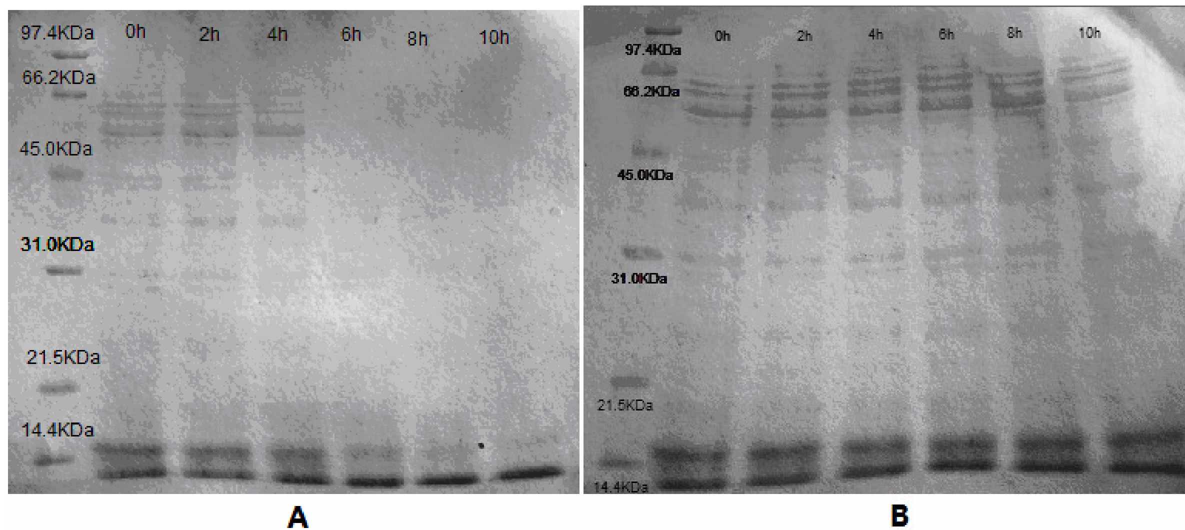


Figure.7 SDS-PAGE pattern of salt-soluble protein in sourdough during fermentation. A- SYL dough, B- SY dough



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