Dynamics of Early Fermentation of Italian Ryegrass (*Lolium multiflorum* Lam.) Silage

Tao Shao[1], N. Ohba, M. Shimojo and Y. Masuda*

Division of Animal Science, Department of Animal and Marine Bioresource Sciences

Graduate School of Kyushu University, Fukuoka 812-8581, Japan

ABSTRACT : The dynamics of fermentation were studied with Italian ryegrass ensiled in the laboratory silos. The silos were kept in the room set at 25°C, and then were opened on 0.5, 1, 2, 3, 5, 7 and 14 days after ensiling, respectively. The samples were taken from three silos at each sampling time for chemical analyses. Mono-and disaccharides composition was determined for glucose, fructose and sucrose by high performance liquid chromatography. The Italian ryegrass silage succeeded to achieve lactate type fermentation; high values of lactic acid (85.83 g/kg) and lactic acid/acetic acid at the end of ensiling (14 day), low values of pH (3.74), acetic acid (5.38 g/kg), ethanol (19.20 g/kg) and NH_3-N/Total N (75.84 g/kg), no or only small amounts of butyric acid, valeric acid and propionic acid. The fermentation dynamics showed a fast and large pH decrease caused by a fast and large production of lactic acid during the first 5 days. Mono-and disaccharides composition largely decreased within initial 0.5 day (12 h) of ensiling. Sucrose disappeared rapidly within initial 0.5 day of ensiling, and fructose and glucose contents showed an initial rise during ensiling, and then decreased gradually. These indicated that the enzymes of plant tissue were active within 2 days of ensiling, which caused the initial rise in fructose and glucose from the hydrolysis of sucrose and fructans. After 5 days of ensilage, glucose was consumed completely, suggesting that glucose was the first fermentation substrate. After 2 days of ensiling, sum amounts of lactic acid and remaining mono-and disaccharides proved to be larger than the quantity of mono-and disaccharides in the initial grass. From the facts mentioned above, it was suggested that considerable amounts of lactic acid were produced from some other substrate such as fructans than initial mono-and disaccharides. (*Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1606-1610*)

INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam.), abbreviated to IR, is one of the most important forage crops. IR is now widely distributed through temperate areas of the world, and generally regarded as the basis of grassland improvement because of its high nutritional value, digestibility, and well ensiling characteristics (Breese, 1983). IR is a major silage crop also in Japan and has been widely used for silage making.

It is well known that the conservation of forage crops by ensiling is based on natural fermentation in which the epiphytic lactic acid bacteria convert sugars under anaerobic conditions into lactic acid. As a result the pH decreases and the silage is preserved. During the early stage of ensiling, air is still present in the silage and this enables plant respiration and aerobic microbial activity to take place: this leads to loss of both nutritive material and fermentation substrates. When pH decreases by lactic acid bacteria under anaerobic condition, the silage is stabilized and the grass can be maintained for a long period. The rate and efficiency of acid production in the first stage of fermentation by the epiphytic lactic acid bacteria are important factors in efficient silage making (Weinberg et al., 1988), thereafter the initial fermentation plays an important role in determining the quality of silage.

Of the common temperate species, IR has the highest water soluble carbohydrates (WSC) content and is often ensiled without the use of additives and resulting in a quality silage (McDonald et al., 1991; Rooke and Kafilzadeh, 1994). The purpose of the present work was to study fermentation quality in the early stage of ensilage of IR in relation to changes in concentrations of mono-and disaccharides.

MATERIALS AND METHODS

Silage making

IR was sown on October 15, 1999, in the experimental field of Kyushu University, Hakozaki, Fukuoka, Japan. The initial growth of IR was harvested at the internode elongation stage using a hand sickle on April 6, 2000. The harvested IR was chopped into approximately 1 cm length with a forage cutter. Eighty grams of chopped grass were immediately packed into a plastic laboratory silo (100 ml liter capacity) in triplicates, followed by being sealed with a screw top and stored in the room kept at 25°C. The silos were opened on 0.5, 1, 2, 3, 5, 7 and 14 days after ensiling.

Chemical analyses

The chopped grass was immediately collected for the determination of dry matter (DM), total nitrogen (TN), crude protein (CP) and mono-and disaccharides (fructose, glucose and sucrose) contents.

After the silos were opened and the contents were mixed thoroughly, 40 grams of the sample were taken from each silo. This was followed by adding 80 grams of distilled water and macerating at 4°C for 24 h. Then, the extracts were filtered through two layers of cheesecloth and a filter paper (Toyo No. 5A). The filtrate was stored at -20°C prior to chemical analyses. The filtrate was used for determining pH, ammonia-N (AN), lactic acid (LA), ethanol, and volatile fatty acids (VFAs). The pH of silages was measured using a glass electrode pH meter (Horiba Co, Japan). TN was analyzed by the Kjeldahl method (AOAC, 1984) and AN with an ammonia electrode (Model IM-22P, Toa Electronics Ltd, Japan). Crude protein was determined with 6.25 multiplied by TN. The LA content was determined using the method of Barker and Summerson (1941), and VFAs and ethanol with gas chromatography (Shimadzu GC-17A, Japan, with 12 m capillary column, condition: column temperature 100°C, injection temperature 250°C). The DM contents of the fresh material and silages were determined by drying in an oven at 60°C for at least 48 h (AOAC, 1984). Mono-and disaccharides of the grass and silages were determined by high performance liquid chromatography (HPLC) as follows; freeze-dried samples were ground to pass through a 1-mm screen and samples (200 mg) were extracted with 80% ethanol (10 ml) on a blockheater at 80°C for 5 h, the ethanol extracts were delivered to centrifugal tube and centrifuged for 15 min at 10,000 g using a supercentrifugal machine. The supernatants were dried for one night at 80°C. The dried samples were dissolved with acetonitrile (75%) and passed through a membrane filter (0.45 μm) and analyzed by HPLC system with NH2P-50 Column (SHOKO. CO., Ltd, Japan). The mobile phase was the degassed 75% CH_3CN: 25% H_2O at a flow rate of 1.0 ml/minute, and sugars were detected with a differential refractometer (Shimadzu, RID-10A, Japan).

Statistical analysis

All data were analyzed statistically by one-way analysis of variance (ANOVA) using a commercially available package (SAS, 1985). Statistical significance among storage periods for each item was determined by Fisher's least significant difference.

RESULTS

Characteristics of the grass before ensiled are shown in Table 1. It showed 149.53 g/kg for DM, 63.75 g/kg for

Table 1. Characteristics of Italian ryegrass before being ensiled (g/kg DM)

Dry matter	Crude protein	Fructose	Glucose	Sucrose	Mono-and disaccharides
149.53	63.75	23.55	14.22	14.64	52.41

crude protein, 23.55 g/kg for fructose, 14.22 g/kg for glucose, 14.64 g/kg for sucrose and 52.41 g/kg for mono-and disaccharides.

The fermentation characteristics of IR silage are presented in Table 2. The major fermentation products were LA, acetic acid (AA) and ethanol. There was a fast and large reduction ($p<0.05$) in pH of the silages just after 1 day of ensiling, a sharp decrease ($p<0.05$) to 3.87 on 5th day, and then remained almost constant until the day 14 of ensiling. The LA content increased significantly ($p<0.05$) after the first day of ensiling, reaching the highest value (138.83 g/kg) on day 5, and then decreased significantly ($p<0.05$) to 85.83 g/kg at the end of the period.

The AA content showed a significant ($p<0.05$) increase after the first day of ensiling, reaching the peak (6.40 g/kg) on day 3, and then varied between 3.67 and 5.38 g/kg. The ethanol content increased significantly ($p<0.05$) after 2 days of ensiling, and reached a highest concentration (38.22 g/kg) on day 5, and then decreased significantly ($p<0.05$) to 19.20 g/kg at the end of the experiment.

The butyric acid (BA), valeric acid (VA) and propionic acid (PA) were detected in no or only small amounts over the ensiling period. Total organic acids content increased significantly ($p<0.05$) after 2 days of ensiling, reaching the highest value on day 5, followed by an insignificant decrease ($p>0.05$). Lactic acid/acetic acid (LA/AA) increased gradually and reached the highest value on day 5 ($p<0.05$), and then tended to decrease. It showed high values in the full fermentation course. AN/TN increased significantly ($p<0.05$) after 2 days of ensiling and continued to increase to the highest value (75.84 g/kg) at the end of ensiling. DM content did not change greatly up to 14 days of ensiling.

Changes in contents of mono-and disaccharides during ensiling are shown in Table 3. Total mono-and disaccharides decreased largely within initial 12 h of ensiling to 43.19 g/kg as compared with the fresh grass (52.41 g/kg), and then showed a gradual decrease until the end of ensiling. Fructose showed an initial rise within 3 days of ensiling from 23.55 g/kg of the fresh grass to 26.00 g/kg, and then gradually decreased to 8.73 g/kg at the end of the experiment. Glucose also tended to increase within 12 h of ensiling from 14.22 g/kg of the fresh grass to 16.49 g/kg, and then showed a large decrease ($p<0.05$) between 1 and 2 days (from 13.71 g/kg to 6.28 g/kg), approaching 0 (0.46 g/kg) on day 5. Sucrose disappeared rapidly within only initial 12 h of ensiling to 0.45 g/kg as compared with the fresh grass (14.64 g/kg).

Table 2. Changes in fermentation quality of Italian ryegrass silage in the early fermentation (g/kg DM)

Item	Storage periods (days)						
	0.5 day	1 day	2 days	3 days	5 days	7 days	14 days
pH (SD)	5.98 (0.13)[d1)]	5.99 (0.03)[d]	4.60 (0.15)[c]	4.06 (0.05)[b]	3.87 (0.04)[a]	3.77 (0.04)[a]	3.74 (0.02)[a]
Dry matter (SD)	144.39 (0.90)[a]	140.37 (3.64)[a]	140.01 (2.39)[a]	145.29 (3.12)[a]	138.90 (0.38)[a]	143.65 (0.33)[a]	143.35 (5.25)[a]
Ethanol (SD)	20.34 (3.89)[a]	21.80 (1.41)[ab]	23.54 (0.24)[ab]	27.91 (1.30)[b]	38.22 (9.76)[c]	22.74 (3.83)[ab]	19.20 (0.85)[a]
Lactic acid (SD)	2.29 (0.74)[a]	4.98 (0.47)[a]	58.23 (9.87)[b]	86.67 (4.04)[b]	138.83 (36.16)[c]	87.73 (25.21)[b]	85.83 (14.86)[b]
Acetic acid (SD)	0.71 (0.18)[a]	0.53 (0.20)[a]	3.00 (0.72)[b]	6.40 (1.21)[d]	4.21 (1.66)[bc]	3.67 (1.03)[b]	5.38 (1.21)[cd]
Butyric acid (SD)	0.02 (0.04)[a]	0.00 (0.00)[a]	0.01 (0.02)[a]	0.01 (0.02)[a]	0.01 (0.02)[a]	0.02 (0.01)[a]	0.01 (0.01)[a]
Valeric acid (SD)	0.07 (0.12)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]
Propionic acid (SD)	0.16 (0.27)[a]	0.00 (0.00)[a]	0.07 (0.12)[a]	0.06 (0.11)[a]	0.11 (0.09)[a]	0.11 (0.08)[a]	0.07 (0.06)[a]
Total organic acids (SD)	3.25 (1.28)[a]	5.52 (0.57)[a]	61.31 (10.45)[ab]	93.14 (5.14)[bc]	159.28 (30.11)[c]	91.53 (25.42)[bc]	108.49 (25.36)[bc]
Lactic acid/Acetic acid (SD)	3.28 (0.96)[a]	10.06 (3.15)[ab]	19.66 (1.96)[bc]	13.78 (1.91)[abc]	36.04 (16.17)[d]	25.09 (8.82)[cd]	19.45 (4.48)[bc]
NH_3-N/TN (SD)	21.67 (3.85)[a]	21.55 (2.31)[a]	35.16 (4.88)[a]	64.13 (10.14)[bc]	55.53 (18.82)[b]	57.56 (8.95)[b]	75.84 (15.12)[c]

[1)] Values followed by different letters in the same row show significant differences at $p < 0.05$.

Table 3. Changes in contents of mono-and disaccharides of Italian ryegrass silage in the early fermentation (g/kg DM)

Item	Storage periods (days)						
	0.5 day	1 day	2 days	3 days	5 days	7 days	14 days
Fructose (SD)	26.25 (1.50)[bc1)]	29.22 (1.43)[bc]	34.80 (16.48)[c]	26.00 (7.84)[bc]	15.58 (5.01)[ab]	16.68 (7.04)[ab]	8.73 (1.29)[a]
Glucose (SD)	16.49 (1.16)[b]	13.71 (4.88)[b]	6.28 (5.73)[a]	4.24 (5.99)[a]	0.46 (0.16)[a]	0.16 (0.15)[a]	0.00 (0.00)[a]
Sucrose (SD)	0.45 (0.71)[a]	0.03 (0.06)[a]	0.21 (0.30)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.20 (0.00)[a]
Mono-and disaccharides (SD)	43.19 (0.41)[b]	42.96 (6.23)[b]	41.29 (22.49)[b]	30.23 (13.81)[ab]	16.04 (4.85)[a]	16.84 (7.02)[a]	8.93 (1.28)[a]

[1)] Values followed by different letters in the same row show significant differences at $p < 0.05$.

DISCUSSION

Results obtained in the present study showed a good fermentation quality as judged from the report of Catchpoole and Henzell (1971); a low pH value (3.74), high LA content (85.83 g/kg), low contents of AA (5.38 g/kg), ethanol (19.20 g/kg) and AN/TN (75.84 g/kg), with no or only small amounts of BA, VA and PA at the end of ensiling (Table 1).

It was shown in 14 day-fermentation dynamics study of IR silage that the rate and extent of reduction in pH were large within initial 5 days of ensiling. This was mainly caused by a rapid and intensive production of LA between 1 and 5 days. During the 14 days of fermentation LA continued to be the major fermentation product with a small production of AA, resulting in the high value of LA/AA over the storage periods (from 3.28 to 19.45). These indicate that acidification was initiated by homofermentative lactic acid bacteria (LAB), and this was dominant during fermentation course. The dominance of homofermentative LAB in the early stage of ensiling in non-additive-treated IR silages is in agreement with that reported by McDonald et al. (1991) and Rooke and Kafilzadeh. (1994). However, after 5 days of ensiling, LA and LA/AA showed a significant decreases between 5 and 14 days, indicating that there was a significant shift from homofermentative to heterofermentative activity. This finding is also in agreement with other studies as reviewed by (Beck, 1972). Beck (1972) also found that, in well-preserved silages, acidification was initiated by homofermentative strains, but after only 4 days 85% of the strains were heterofermentative, suggesting the eventual dominance of heterofermentative strains due to their greater tolerance to acetic acid (Beck, 1978).

Total organic acids content showed a significant ($p < 0.05$) increase during ensilage, and reached the peak on day 5. This indicates that greatest fermentation activity occurred within the initial 5 days of ensiling. It has been well known that cell breakdown and the resultant release of the plant juices are prerequisite for the production of significant amounts of LA during ensiling. The water activity increases with the increase in moisture content (Greenhill, 1964a,b,c). Gibson et al. (1961) reported that the mondern precision-chop forage harvester can chop herbage into short particle length and ensure a rapid plant sap liberation which stimulates the LAB growth, especially homofermentative LAB in the early fermentation. We harvested IR at the immature stage (internode elongation stage) with high moisture content and cut at once into 1 cm and crushed, thus it was considered that cell breakdown and release of the plant juices were more easy. Therefore, the ensiled grass produced LA rapidly just after ensiling. The

increases in AA and ethanol contents during the fermentation suggested the activity of some heterofermentative LAB or yeast. However, ethanol content was higher than AA during the storage period probably due to the survival of some yeasts throughout the ensilage period (Weinberg et al., 1988; Driehuis et al., 1997; Henderson and McDonald, 1971). The laboratory silo was sealed, but there was still a little amount of O_2 in the silo and sufficient fermentable sugars were available for yeasts to maintain their metabolism (Ashbell and Kashanchi, 1987).

The BA, VA and PA were detected in no or only small amounts over the ensiling period. This attributed to a rapid reduction in pH because of the rapid production of LA, restricting the growth of clostridia and other bacteria (Catchpoole and Henzell, 1971; McDonald et al., 1991; Henderson, 1993).

AN/TN increased remarkably during the fermentation, but the content was less than 80 g/kg at the end of ensiling. This was associated with the production of some ammonia from other sources such as the breakdown of nitrates and nitrites, the action of plant enzymes and enterobacteriaceae (Seale, 1986). DM content did not change greatly up to 14 days of ensilage, because the laboratory silos were in anaerobic conditions and had no seepage loss.

Fructose content tended to increase within 3 days of ensiling and glucose content also showed an initial rise, whereas sucrose rapidly disappeared in short time within 12 h. These findings agree with those of Masaki and Ohyama (1979). It can be considered that the increase in fructose or that in glucose was due to the hydrolysis of sucrose and fructans, suggesting that residual plant enzymes were active in the early period of ensiling (Clark, 1974; Bousset et al., 1972; Gouet et al., 1970). Glucose decreased more rapidly to 0.46 g/kg on the 5th day than fructose, which indicated the role of glucose as the first fermentation substrate (Hattori et al., 1993).

Total mono-and disaccharides showed a large decrease within initial 12 h of ensiling (from 52.41 to 43.19 g/kg), and then decreased gradually until the end of the ensiling. This was partly due to the respiration loss during initial 12 h of fermentation, which was similar to that found by other workers (Wylam, 1953; Carpintero et al., 1969; Seale, 1986). Sum amounts of LA and remaining mono-and disaccharides proved to be larger than the quantity of mono- and disaccharides in the material. From the facts mentioned above, it is suggested that considerable amounts of LA were produced from some other substrate than initial mono-and disaccharides (Masaki and Ohyama, 1979). This is not surprising as Italian ryegrass is a temperate grass in which fructans are the most abundant source of the WSC, and in the initial stage of ensilage most of the fructans were hydrolyzed into fructose and glucose.

In conclusion, this study showed that the silage from IR with high moisture content had a good fermentation characteristic, where active LA fermentation took place in the initial stage of ensilage due to the activity of homofermentative LAB that dominate in the earlier stage rather than the later stage of ensilage. These results can be explained by a rapid plant sap liberation which stimulates the homofermentative LAB growth (Greenhill, 1964a,b,c). Changes in the mono-and disaccharides content suggested the high activity of the plant enzymes within initial 2 days of ensiling. Glucose was the first fermentation substrate. Our initial motives intended to compare the natural fermentation characteristics of IR with those of tropical grasses, since they are different in chemical and physical characteristics (Smith, 1973). In the future work, we will study the dynamics of early fermentation of guineagrass silage.

REFERENCES

AOAC. 1984. Official Methods of Analysis. 14th edn. Association of Official and Analytical Chemists Arlington, Virginia-2221.

Ashbell, G. and Y. Kashanchi. 1987. In-silo losses from wheat ensiled in bunker silos in subtropical climate. J. Sci. Food Agric. 40:95-103.

Barker, S. B. and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138:535-554.

Beck, T. 1972. Die quantitative und qualitative Zusammensetzung der Milchsauerebakterienpopulation im Garfutter. Land-wirtschaftliche Forschung. 27:55-63.

Beck, T. 1978. The microbiology of silage fermentation. In: Fermentation of Silage-a Review Ed. McCullough and M. E. Iowa: National Feed Ingredients Association. pp. 61-115.

Bousset, J., N. Bousset-Fatianoff, Ph., Gouet and M. Contrepois. 1972. Annales de biologie Animale, Biochimie et Biophysique. 12:453-477.

Breese, E. L. 1983. Exploitation of genetic resource through breeding: Lolium species. In: Genetic Resources of Forage Plants (Ed. G. Mclover and R. A. Bray). CSIRO 1983, Australia. 275-288.

Carpintero, M. C., A. J. Holding and P. McDonald. 1969. Fermentation studies of Lucerne. J. Sci. Food Agric. 29:497-505.

Catchpoole, V. R. and E. F. Henzell. 1971. Silage and silage making from tropical herbage species. Herb. Abstr. 41:213-219.

Clark, B. J. 1974. Ph. D. Thesis, University of Edinburgh.

Driehuis, F., P. G. Van. Wilkselaar, A. M. Van. Vuuren and S. F. Spoelstra. 1997. Effect of a bacteria inoculant on rate of fermentation and chemical composition of high dry matter grass silages. J Agric. Sci. Cambridge. 128:323-329.

Gibson, T., A. C. Stirling, R. M. Keddie and R. F. Rosenberger. 1961. Bacteriological changes in silage as affected by laceration of the fresh grass. J. Appl. Bacteriol. 24:60-70.

Gouet, Ph., N. Fatianoff and J. Bousset. 1970. Compte Rendu del Academie de Sciences, Paris. 270:1024-1027.

Greenhill, W. L. 1964 a. Plant juices in relation to silage

fermentation. I. The role of the juice. J. Br. Grassl. Soc. 19:30-37.

Greenhill, W. L. 1964b. Plant juices in relation to silage fermentation. II. Factors affecting the release of juices. J. Br. Grassl. Soc. 19:231-236.

Greenhill, W. L. 1964 c. Plant juices in relation to silage fermentation. III. Effect of water activity of juice. J. Br. Grassl. Soc. 19:231-236.

Hattori, I., S. Kumai and R. Fukumi. 1993. The effect of saccharide additives on the fermentation quality of silage. J. Japan. Grassl. Sci. 39:326-333.

Henderson, A. R. and P. McDonald. 1971. Effect of formic acid on the fermentation of grass of low dry matter content. J. Sci. Food Agric. 22:157-163.

Henderson, N. 1993. Silage additives. Anim. Feed Sci. Technol. 45:35-36.

Masaki, S. and Y. Ohyama. 1979. Changes in sugars during ensilage-production of lactic acid and volatile fatty acids. Jap. J. Zootech. Sci. 50(5):280-287.

McDonald, P., A. R. Henderson and S. J. E. Heron. 1991. The Biochemistry of Silage. 2nd ed. Cambrian Printers Ltd.

Aberystwyth. pp. 184-236.

Rooke, J. A. and F. Kafilzadeh. 1994. The effect upon fermentation and nutritive value of silages produced after treatment by three different inoculants of lactic acid bacteria applied alone or in combination. Grass and Forage Sci. 49:324-333.

SAS. Institute Inc. 1985. SAS/STAT User`s Guide: Version 6. 4th edn. SAS Institute Inc., Cary, North Carolina.

Seale, D. R. 1986. Bacterial inoculants as silage additives. J. Appl. Bacteriol., Symp. Suppl. 9S-26S.

Smith, D. 1973. The nonstructural carbohydrates. In : Chemistry and Biochemistry of Herbage (Ed. G. W. Butter and R. W. Bailey). Academic press, New York. Volume. 1:105-155.

Weinberg, Z. G., G. Ashbell and G. Azrieli. 1988. The effect of applying lactic acid bacteria at ensilage on the chemical and microbiological composition of vetch, wheat and alfalfa silages. J. Appl. Bacteriol. 64:1-7.

Wylam, C. 1953. Analytical studies on the carbohydrates of grasses and clovers. III. Carbohydrate breakdown during wilting and ensilage. J. Sci. Food Agric. 4:527-531.

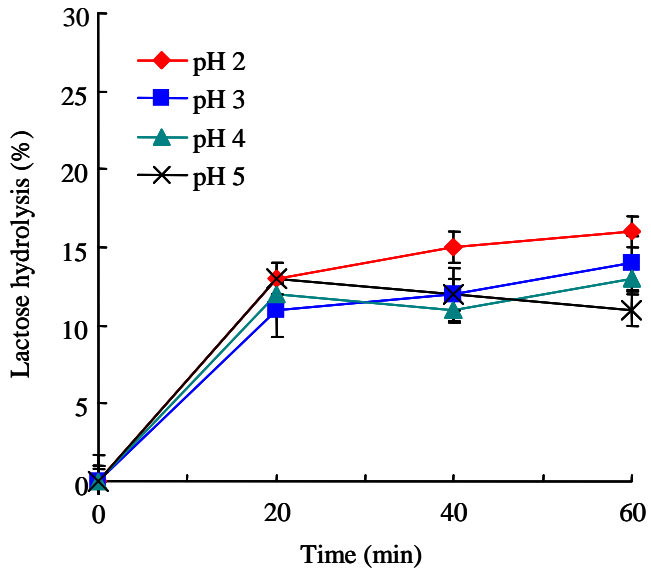| 14 days |
| --- |
| 3.74 (0.02) a |
| 143.35 (5.25) a |
| 19.20 (0.85) a |
| 85.83 (14.86) b |
| 5.38 (1.21)cd |
| 0.01 (0.01) a |
| 0.00 (0.00) a |
| 0.07 (0.06) a |
| 108.49 (25.36) bc |
| 19.45 (4.48) bc |
| 75.84 (15.12) c |

Figure 2. Hydrolysis pattern of lactose form PGMS microcapsules as a function of time during incubation in a simulated gastric fluid.