

PRODUCTION AND PROPERTIES OF RENNET FROM BUFFALO CALVES ABOMASAM

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ABSTRACT

Present study was planned to produce and evaluate liquid rennet from buffalo calves abomasa. Out of 30 abomasa collected, 20 samples were selected for the study and analyzed for proteolytic activity, clotting activity, productivity, shelf life, pH and sensory evaluation. Non protein nitrogen (NPN) contents of rennet coagulated milk ($1.33 \pm 0.02\%$) were significantly higher ($P < 0.01$) than that of raw milk ($0.25 \pm 0.001\%$) indicating the significant proteolytic activity of rennet. Clotting activity (per unit volume) of group A abomasa (22437 ± 1593.60) was higher than that of group B abomasa (9948.10 ± 647.74). Curd yielded by fresh laboratory made rennet (LMR) ($32.2 \pm 0.24\%$) was significantly higher ($P < 0.01$) than that of stored LMR ($29.3 \pm 0.11\%$) and commercial rennet ($28.20 \pm 0.13\%$). All the rennet samples were active up to 3 months storage, whilst 15% samples became inactive after 6 months storage periods. Strength (per unit volume) of fresh LMR was comparatively high (17256 ± 1599.40) and decreased gradually with the period of time i.e. 3m stored rennet (12430 ± 1167.90) and 6m stored rennet (7106.8 ± 539.78). Clotting activity of LMR on buffalo milk (14716 ± 3911.70) was found to be non significant ($P > 0.05$) than that of cow milk (11014 ± 2507.80). pH value of stored LMR (5.75 ± 0.02) was significantly higher ($P < 0.01$) than that of fresh LMR (5.47 ± 0.02) with the period of time. Scores rated for appearance (4.06 ± 0.001) and odor (4.22 ± 0.01) of fresh rennet were significantly different ($P < 0.05$) from that of stored rennet (3.55 ± 0.0004 and 4.07 ± 0.001 , respectively). While there were no difference ($P > 0.05$) in the score of color intensity of fresh (3.93 ± 0.02) and stored rennet (3.88 ± 0.02). It was concluded from the study that liquid rennet can be prepared at local level with adequate clotting activity, better productivity and reasonable shelf life.

Key words: Rennet, Calf abomasa, Chymosin, Clotting activity.

INTRODUCTION

Cheese is generally manufactured by clotting the milk with a milk clotting enzyme (rennet). The most common milk clotting enzyme, used in many cheese varieties worldwide, is calf rennet obtained from 10 to 30 days old milk-fed calves (Yadav *et al.*, 1993). It consists of mainly the rennin (chymosin) and pepsin, former being principally responsible for milk clotting and the later for proteolysis. The rennet from the young milk fed calf is rich in rennin (chymosin), while, rennet from the older bovine is rich in pepsin (Akin, 1996). In addition to the calf many other animals including the lamb, goat, pig, bovine, rabbit and hen contain the enzyme chymosin in their digestive systems (Metin *et al.*, 1998). Alternative substances used to clot milk have a vegetable origin and are manufactured from non-animal sources. Vegetable sources include the cheese-rennet herb, 'Ladies bedstraw' (*Galium verum*). Fungal enzymes produced by *Rhizomucor miehei*, *Mucor pusillus* and *Cryphonectria parasitica* are also available for clotting milk. These are less sensitive to temperature changes than rennet derived from cattle abomasums. Clotting agents prepared from other vegetable material such as figs, pineapple or bacteria are less successful rennet substitutes. Genetically

engineered chymosin (the main enzyme present in calf rennet) has been produced from yeast (*Kluveromyces lactis*), bacteria (*Escherichia coli*) and fungi (*Aspergillus niger*). Rennet is mainly used in hard cheese making and little is used in the manufacture of soft cottage cheese or fromage frais. Lactose (milk sugar), galactose, lactulose and ethanol are by-products of cheese manufacture derived from whey. Whey is the liquid end-product produced when rennet is added to whole milk, partially skimmed or skimmed milk (milk from which the butter fat/cream is removed by centrifugation). Lactose prepared from whey has an important usage during the manufacture of pharmaceutical and biological products and is also used in confectionery. According to lactose manufacturers, approximately 90% of lactose for use in the pharmaceutical industry is prepared from rennet-derived whey (EMEA, 2002). Whey is used in feed for pigs, possibly other animals and at least historically in infant foods. The other major product of rennet-treated milk is the curd from which cheese is manufactured. A small amount of rennet is sold for cooking. After milk is treated with rennet 50-90% of it partitions with the whey and 10- 50% with the curds where it continues to function during maturation of the cheese (Anonymous, 2002). Since cheese manufacturing is getting great importance in national dairy industry, there is need to produce

indigenous type of rennet, as commercially available rennet is too costly to afford. No reliable research work so far has been found in the past in this respect. Thus, the present study merits producing rennet from buffalo calf stomach and evaluating its quality characteristics.

MATERIALS AND METHODS

Collection and processing of milk and abmosa: Raw milk of buffalo and cow was obtained from the Livestock Experiment Station, Department of Livestock Management, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. Buffalo calves Abomasa were collected from the butchers in the vicinity of Hyderabad. Commercial rennet powder (Christan Hansen's Laboratories Ltd., Copenhagen) was used in equal dilution to coagulate the milk for comparison purpose. Similarly different chemicals like Sodium benzoate (Sigma-Aldrich) was used as preservative during rennet production. Benzoic acid (Sigma-Aldrich) was used to lower the pH of the solution. Disodium phosphate (Sigma-Aldrich) was used to enhance the pH of the extract. Food grade Sodium chloride (Sigma-Aldrich) was purchased from Alberuni Scientific Store, Hyderabad. A wooden-wire- mesh cabinet (locally prepared) was used to dry the air filled abomasa. Plastic tubs purchased from the local market of Tandojam were used for soaking strips of dried abomas. A pH meter (Hanna Instrument, model HI 8471, Italy) was used to examine rennet pH value. Water bath was used to inoculate the samples during the rennet strength and curd determination. Micro Kjeldhal Digestion unit was used to digest the samples for determination of non protein nitrogen contents. Micro Kjeldhal Distillation unit was used to distillate the samples during nitrogen determination. Titration kit was used for the titration of distilled samples during nitrogen determination. Electrical weighing balance (Mettler PJ 300, Mettler Instruments AG Switzerland) was used to take weight of curd as well as of chemicals during analysis.

A total 30 abomasa of buffalo calves were collected from the butchers in the vicinity of Hyderabad and brought to the Laboratory of Dairy Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam for the production of liquid rennet. Among 30 samples 10 (33.33%) abomasa were found to be filled with curdled material (ingesta). While 13 (43.33%) abomasa seemed to be filled with mixture of curdled material and ground grassed material (ingesta / digesta). However, the remaining 7 (23.33%) abomasa showed liquefied ground grassed (digesta) as a filled material, which were not suitable for rennet production. Although 23 abomasa (i.e. ingesta and ingesta/ digesta) were suitable for rennet making but to avoid the heterogeneity in sampling, 10

abomasa (from each of ingesta and ingesta/ digesta) were selected for the present study.

Preparation of rennet: Rennet was prepared according to the method as described by Lambert (1988). The basic steps of rennet making are shown in flow chart (Figure-3.1). After removing the internal contents, abomasa were washed with tap water internally while their veins and fat contents were removed externally. Then they were inflated with air just like a balloon and dried in wooden- wire mesh cabinet placed in an open air. After drying, each abomasam was cut into very thin strips separately and soaked in 700 ml solution of the 12 % NaCl and 1% sodium benzoate solution individually. The pH of the solution containing abomasal strips was reduced to 4.3 with benzoic acid. The solution was stored at 35^oC for 72 hrs. The pH of the mixture was raised to 5.6 with disodium phosphate. The extract was filtered with muslin cloth and caramel-like color liquid rennet (approximately 500 ml) was obtained from the individual sample.

Estimation of rennet activity: Proteolytic activity of rennet was evaluated by comparing non protein nitrogen (NPN) contents of raw milk and rennet coagulated milk. NPN content of raw milk and rennet coagulated milk was determined according to the method of International Dairy Federation (IDF, 1993). Sample (10ml /1g) was taken into a pre-weighed conical flask and re-weighed (nearest 0.1mg). Trichloro acetic acid (TCA) solution (40ml) was added to the flask, and contents with flask weighed. Solution was swirled to mix and left to stand approximately 5min to allow the precipitate settle. Contents of the flask were filtered through the filter paper and filtrate was collected in the clean, dry conical flask. Mixed filtrate (20ml) was digested using Micro-Kjeldhal digester in the presence of catalyst (0.2g copper sulfate and 2g sodium sulfate/potassium sulfate) where sulfuric acid (20-30 ml) was used as an oxidizing agent. The digested sample was diluted with distilled water (250 ml). Then 5 ml portion from the diluted sample was distilled with NaOH (40%) using Micro-Kjeldhal distillation unit where steam was distilled over 2% boric acid (5 ml) containing an indicator for 3 minutes. The ammonia trapped in boric acid was determined by titrating with 0.1N HCl. The nitrogen percentage was calculated using the following formula:

$$N (\%) = 1.4 (V1 - V2) \times \text{normality of acid} \times 250 / \text{Weight of sample taken} \times \text{Sample used for distillation}$$

Protein equivalent of non protein nitrogen content was determined by conversion of nitrogen percentage to protein assuming that all the nitrogen in milk was presented as protein i.e. protein percentage = N% x CF. Whereas CF = 100/N % in protein of milk and dairy products i.e. 15.66. Strength of fresh and stored liquid rennet was analyzed according to the method as described by Lambert (1988). Buffalo milk (500ml) was

taken in to Erlenmyer flask and heated up (35° C) in a water bath. Then 10 ml diluted liquid rennet (1:10) was dissolved in the milk by swirling the flask continuously. As the flocculation started the time was noted. Then strength was determined by following formula: $Strength = 2400 \times 500 / Time\ in\ seconds \times 1$

pH values of liquid rennet samples were determined using pH meter. Sensory attributes of rennet were evaluated according to the method as reported by Cakmakci and Boroglu (2004). A panel of 5 assessors was drawn from M.Sc. students of Department of Dairy Technology. Assessors evaluated the rennet samples with respect to appearance, color and odor.

RESULTS

Proteolytic activity of laboratory made rennet (LMR) was studied by comparing non protein nitrogen (NPN) content of raw milk, and rennet coagulated milk. The results revealed that there was significant ($P < 0.001$) difference between the NPN % of raw milk and rennet coagulated milk. Average NPN % of raw milk was found as $0.25 \pm 0.001\%$ (range, 0.22 to 0.29%), contrary to average NPN % of rennet coagulated milk of $1.33 \pm 0.02\%$ (range 1.19 to 1.45%). LSD comparison of means at rejection level of 0.05 revealed that there was significant difference ($P < 0.01$) between the two means. Clotting activity (strength) of laboratory made rennet samples extracted from group A and B abomasa was determined. Average clotting activity of rennet from group A and group B abomasa was observed, as 22437 ± 1593.60 (range, 13333.0 to 30000.0) and 9948.10 ± 647.74 (range 6666.0 to 12000.0), respectively. Statistical analysis (ANOVA) indicated highly significant differences ($P < 0.001$) between the clotting activity of rennet extracted from group A and B abomasa. Whilst LSD comparison of means at the rejection level of 0.05 revealed that both the means were significantly different from each other ($P < 0.05$). Curd yield obtained by coagulating milk with laboratory made rennet (fresh and stored rennet) and commercial rennet was calculated in the form of curd yield and the results are expressed in Tables. Curd yield (%) varied between 30.46 to 33.60% during renneting milk with fresh LMR; in between 27.30 to 29.0% by clotting milk with stored (3M) LMR and in a range of 29.30 to 30.55% by coagulating milk with commercial rennet. While the mean values ($32.2\% \pm 0.24\%$) of curd obtained from fresh rennet coagulated milk was highest followed by commercial rennet coagulated milk ($29.87\% \pm 0.11\%$) and stored rennet coagulated milk ($28.24\% \pm 0.13\%$). Statistical analysis (ANOVA) performed on the data of curd yield revealed highly significant differences ($P < 0.001$) between fresh, stored and commercial rennet coagulated milk. However, LSD comparison of means at rejection level of 0.05 separated the data into three groups in which

means were significantly different ($P < 0.01$) from one another.

Shelf life of laboratory made rennet (LMR) was observed by comparing the strength of fresh, three months and six months stored LMR, and results are mentioned in Tables. All the rennet samples were active up to 3 months storage, whilst 15% samples became inactive after 6 months storage periods. Strength of fresh LMR was comparatively high 17256 ± 1599.40 puv (8571 to 30000 puv) and decreased gradually with the period of time i.e. 3 months stored rennet 12430 ± 1167.90 puv (6054 to 21000 puv) and 6 months stored rennet 7106.8 ± 539.78 puv (4000 to 12610 puv). Furthermore, one way analysis of variance reveals highly significant differences ($P < 0.001$) between strength of fresh, 3 and 6 months stored rennet, whilst LSD comparison of means at rejection level of 0.05 revealed that all the means of strength were significantly different ($P < 0.05$) from one another. Suitability of liquid rennet from buffalo calves abomasa was determined by comparing the clotting activity of rennet on cow and buffalo milk, and the results are depicted in Tables. The results indicate that the clotting activity on cow milk varied between 6666.70 to 20690 puv and buffalo milk varied from 8571.40 to 30000 puv. While mean clotting activity of rennet on buffalo milk (14716 ± 3911.70) was found slightly greater than mean clotting activity on cow milk (11014 ± 2507.80). However, results of analysis of variance showed no significant difference ($P > 0.05$) between the means.

pH value of fresh and stored rennet ranged from 5.36 to 5.74 and 5.65 to 5.98, respectively (Table 2). Whilst mean pH value of stored way analysis of variance reveals highly significant differences ($P < 0.001$) between pH values of fresh and stored rennet. Whilst LSD comparison of means at rejection level of 0.05 reveals that two means were significantly different from each other ($P < 0.05$). It was observed that score rated for appearance of fresh LMR ranged from 4.01 to 4.15 (mean, $4.06 \pm 8.838E-03$) and stored LMR 3.52 to 3.60 (mean, $3.55 \pm 4.441E-03$) from a total score of 5. One way analysis of variance reveals highly significant differences ($P < 0.001$) between the score rated for appearance of fresh and stored rennet. However, LSD comparison of means at rejection level of 0.05 reveals that two means were significantly different from each other ($P < 0.05$). It was observed that score rated for color of fresh LMR varied from 3.78 to 4.11 (mean, 3.93 ± 0.02) and stored LMR 3.75 to 4.01 (mean, 3.88 ± 0.02) from a total score of 5. Results of analysis of variance showed no significant differences ($P > 0.05$) between the means. Odor of laboratory made rennet was assessed by a panel of assessors. It was observed that score rated for odor of fresh LMR appeared in a range from 4.12 to 4.30 (mean, 4.22 ± 0.01) and stored LMR 3.98 to 4.12 (mean, $4.07 \pm 9.570E-03$) from a total score of 5. One way analysis of

variance reveals significant differences ($P < 0.005$) between score rated for odor of fresh and stored rennet. The means of score rated for odor of fresh and stored rennet were statistically different ($P < 0.05$) from each other.

DISCUSSION

Proteolytic activity of laboratory made rennet (LMR) was analyzed by comparing non protein nitrogen (NPN) contents of raw milk and rennet coagulated milk. Average NPN % of raw milk was found $0.25 \pm 0.001\%$ (range, 0.22 to 0.29%) on contrary to average NPN % of rennet coagulated milk i.e. $1.33 \pm 0.02\%$ (range, 1.19 to 1.45%). The results revealed that there was significant difference between the NPN content of raw milk and rennet coagulated milk. Libouga *et al* (2008) compared the proteolytic and clotting activities of kid rennet and calf rennet for their action on goat (*Capra hircus*) milk and cow (*Bos aurus*) milk. The proteolysis was measured by determining the increase of non-protein nitrogen according to the Kjeldahl method. NPN content increased due to the degradation of various casein primary fractions. Proteolytic activity of calf rennet on goat casein (CN) showed that casein was hydrolyzed to give characteristic breakdown products derived from individual caseins (β -I to β -V from β -CN hydrolysis product from α_s1 CN, para- κ -CN from κ -CN and other degradation products from α_s2 CN (Trujillo *et al.*, 1997). Clotting activity (strength) of laboratory made rennet was analyzed by comparing the strength of rennet samples extracted from group A and group B abomasa. It was observed that average clotting activity (22437 ± 1593.60 puv) of rennet from suckling calves (group A; abomasa containing ingesta) was higher and decreased (9948.1 ± 647.74 puv) with advancing age (group B; abomasa containing ingesta/digesta). Present results are in agreement with Zhang *et al.* (2005) who reported that chymosin activity in abomasums of suckling kids at 5 days of age was significantly ($P < 0.01$) greater than other groups with increasing age. They further reported that the chymosin activity of weaned kids gradually decreased from 10 to 20 days, for kids of the same age. However, the suckling group ranked the highest, the random suckling group the second and the weaned group the last.

Curd yield obtained by coagulating milk with laboratory made fresh rennet was highest ($32.20\% \pm 0.24$) followed by commercial rennet coagulated milk ($29.87 \pm 0.11\%$) and stored rennet coagulated milk ($28.24 \pm 0.13\%$). After milk was treated with rennet, 50-90% partitions with the whey and 10-50% with the curds where it continues to function during maturation of the cheese (Anonymous, 2002). Shelf life of laboratory made rennet was observed and results reflected that all the samples of liquid rennet were active up to three months

storage period, while after six months evaluation 15% rennet samples were found inactive. It was contrary to the findings of Lambert (1988) who reported three months shelf life of liquid rennet. But by comparing the clotting activity of fresh, three months stored and six months stored rennet, the result showed the consistent decline in activity with the passage of time indicating its diminishing shelf life. Maximum clotting activity was noted in fresh rennet (17256 ± 1599.40 puv) followed by 3 months stored rennet (12430 ± 1167.90 puv) and 6 months stored rennet (7106.80 ± 539.78 puv). However, clotting activity of the liquid rennet observed in the present study was within the range of reported activity of liquid rennet (Cakmakci and Boroglu, 2004; Lambert, 1988) i.e. ranged from 5670 to 45450. Suitability of laboratory made rennet (LMR) from buffalo calves abomasa on milk of cow was determined and compared with the clotting activity of buffalo milk. It was noted that mean clotting activity of LMR on buffalo milk (14716 ± 3911.70) was found slightly greater than mean clotting activity of LMR on cow milk (11014 ± 2507.80). The result indicates that more buffalo calf rennet was required to coagulate similar amount of cow milk than that of buffalo milk. However, the result was statistically non-significant ($P > 0.05$). Camel milk require more calf rennet than cow milk to coagulate and relative amount of rennet needed varies widely (Farah and Bachmann, 1987). pH value of laboratory made fresh and stored rennet ranged from 5.36 to 5.74 and 5.65 to 5.98 respectively while mean pH value of stored rennet (5.75 ± 0.02) was noted greater then that of fresh rennet (5.47 ± 0.02). It was in accordance with the study of Cakmakci and Boroglu, (2004) who investigated that pH of liquid rennet varied from 5.08 to 5.82. Results depicted that with the passage of time, pH value of liquid rennet significantly increased while its clotting activity significantly declined. Literature also indicates that clotting activity of rennet decreases as its pH turns towards alkalinity (Elaysed, 2000). Sensory attributes of rennet like appearance, color and odor were assessed by a panel of assessors. Score rated for appearance of fresh and stored Laboratory made rennet ranged, between 4.01 to 4.15 (mean $4.06 \pm 8.838E-03$) and 3.52 to 3.60 (mean $3.55 \pm 4.441E-03$) from a total score of 5 (means rennet was clear and free off suspensions). It was noted that score rated for color of fresh and stored laboratory made rennet ranged, between 3.78 to 4.11 (mean 3.93 ± 0.02) and 3.75 to 4.01 (mean 3.88 ± 0.02) from a total score of 5 (means rennet's typical color i.e. caramel-like color). Results showed that score rated for odor of fresh and stored laboratory made rennet ranged, between 4.12 to 4.30 (mean 4.22 ± 0.01) and 3.98 to 4.12 (mean $4.07 \pm 9.570E-03$) from a total score of 5 (means odor unique to rennet). Statistically, significant difference was observed in the appearance and odor of fresh and stored rennet while, no significant difference was observed in the color

of fresh rennet. The present results on sensory analysis were in line with those of Cakmakci and Boroglu (2004). They made sensory analysis on commercial liquid rennet samples (25) with respect to appearance, color and odor

of liquid rennet samples. They observed that 23 (92%) samples were clear and sediment free in appearance: 11 (44%) samples were of caramel-like color: 17 (68%) samples had odor unique to rennet.

Table 1. Descriptive statistics on activity of commercial and laboratory made rennet.

Variance	Curding % of buffalo milk			Strength (per unit volume)			Clotting activity (per unit volume) on milk	
	Fresh	Stored	Commercial	Fresh	Stored (3m)	Stored (6m)	Cow	Buffao
Minimum	30.46	27.30	29.30	8571.0	6054.0	4000.0	6666.7	8571.4
Maximum	33.60	29.00	30.55	30000.0	21000.0	12610.0	20690	30000
Mean	32.21	28.25	29.87	17256.0	12430.0	7106.8	11014	14716
SE±	0.24	0.13	0.11	1599.4	1167.9	539.9	2507.8	3911.7

Table 2. Descriptive statistics on pH, appearance and color of fresh and stored laboratory made rennet.

Variance	pH		Appearance (score)		color (score)	
	Fresh	Stored	Fresh	Stored	Fresh	Stored
Minimum	5.36	5.65	4.01	3.52	3.78	3.75
Maximum	5.74	5.98	4.15	3.60	4.11	4.01
Mean	5.47	5.75	4.06	3.55	3.93	3.88
SE ±	0.02	0.02	8.838E-03	4.441E-03	0.02	0.02

Conclusions: It was concluded from the present study that shelf life of liquid rennet exceeded the period of six months. With the period of time, pH value of liquid rennet raised. Strength of liquid rennet was declined with the passage of time rennet extracted from the abomasa having milk as a filled material had greater clotting activity than those of abomasa having both milk and grass as a filled material. Appreciable difference was observed in appearance and odor of stored rennet as compared to fresh rennet. Curd yield obtained by renneting milk with fresh liquid rennet was higher than that of commercial rennet.

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