ORIGINAL ARTICLE

REDUCTION IN LACTOSE CONTENT of BUFFALO'S (*Bubalus bubalis*) MILK USING *Aspergillus oryzae*

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ABSTRACT

To determine the reduction of lactose in buffalo's milk after inoculation with *Aspergillus oryzae*, 100 ml of whole buffalo's milk was inoculated with 10 ml spore suspension (1.12 x 10^7 spores/ml) (T2) and another 100 ml milk was added with 10 ml supernatant from previously *A. oryzae*-inoculated milk (T3). These were then incubated for 4 hr at 30°C together with the non-inoculated milk (T1). Significant differences in mean lactose contents and reducing sugar contents (P≤0.05) were observed. The lactose content and reducing sugar content decreased in T2 by 29.71% and 14.20%, respectively. The lactose content of whole buffalo's milk in T3 decreased by 48.77% while the reducing sugars increased by 17.51%. The reduction in lactose and reducing sugars in T2 milk was due to the utilization of lactose as carbon source by the fungal spores for lactase production. The reduction in lactose content and increment in reducing sugars in T3 milk were due to enzymatic action of extracellular lactase (β-galactosidase) produced by the *Aspergillus oryzae*. The results indicate that lactase produced by *A. oryzae* can breakdown lactose in buffalo's milk and *A. oryzae* is a potential source of extracellular enzyme for the production of lactose-free milk.

Key Words: *Aspergillus oryzae*, buffalo, lactase, lactose, milk, reducing sugar

INTRODUCTION

Lactase (β-*galactosidase*) is an enzyme necessary to break down lactose into glucose and galactose once it enters the stomach. It prevents an individual from suffering from lactose intolerance, which causes flatulence, abdominal bloating and diarrhea. Lactase is commercially available in the local market in liquid and dry form. It is also known to contribute to the improvement of the physico-chemical properties of milk by making it more desirable for processing such as increased sweetness, reduced time to reach the desired pH and more ready for fermentation of milk sugars. Despite the known advantages provided by lactase and lactose-free products in the dairy industry, producing such under local conditions is still a difficult task nowadays. Lactase is usually imported from other countries which are capable of adapting highly defined technological equipment. The process of providing lactose-hydrolyzed (lactose-free) milk products in the Philippines is hindered by lack of suitable equipment and cheap commercially available lactase.

Several studies have shown the potential sources of lactase. Enzyme preparations have been available to processors particularly in the food industry during the past several years and may fall into three basic categories: animal-derived preparations, plant-derived preparations and microbiologically-derived preparations (Considine, 2005). Microbial sources allow large quantities of suitable enzyme to be extracted (Palmer, 1985). The bacterial species currently used by the dairy industry for the production of β-galactosidase

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enzyme belong to the genera *Lactobacillus* and *Bifidobacterium* and comprise a limited collection of strains (He *et al.*, 2008). *Aspergillus oryzae* is a fungus noted for its ability to produce lactase. The optimum conditions for ß-galactosidase activity and its activity on cow's milk were previously reported by Tanaka *et al.* (1975) and Park *et al.* (1979), respectively. Rosal (2012) investigated the potential of *A. oryzae* in the production of lactose-free buffalo's milk. *A. oryzae* reduced the lactose content of buffalo's milk by 100% after 4 hr incubation at 27°C. Furthermore, sensory quality of lactose-free buffalo's milk did not differ from the whole milk. The limitation of the experiment was the inability to determine whether the degradation of the lactose content of milk was due to enzymatic activity of the presumed lactase (ß-*galactosidase*) produced by *A. oryzae*.

The current study aimed to determine the reduction of lactose in buffalo's milk due to the presence of lactase (β-*galactosidase*) after inoculation with *A. oryzae* and addition of supernatant from a previously *A. oryzae*-inoculated milk.

MATERIALS AND METHODS

Experimental treatment and design

Buffalo's milk was alloted to three (3) experimental treatments as follows: T1 – whole buffalo's milk (control); T2 – whole buffalo's milk inoculated with *A. oryzae* spore suspension at 1.12 x 107 spores/ml; T3 – whole buffalo's milk added with supernatant from a previously A. oryzae-inoculated milk.

The milk samples for T2 was prepared by directly inoculating 100 ml of whole buffalo's milk with 10 ml of 1.12 x 10^7 spores/ml spore suspension which was previously estimated and standardized. The milk for T3 was prepared by adding 10 ml supernatant from a previously *A. oryzae*-inoculated milk into 100 ml whole buffalo's milk. The supernatant was obtained after centrifugation of the incubated *A. oryzae*-inoculated milk. Treatments 1, 2 and 3 were incubated at 30°C for 4 hr. The experiment was run three times with each run considered as a replicate.

Source of milk

Buffalo's milk used in the experiment was collected from the Philippine Carabao Center, University of the Philippines Los Baños. The milk used all throughout the experiment was bought in a single batch and were pasteurized prior to preparations for the experimental treatments. The milk was subjected to clot-on-boiling and titratable acidity tests, and pH determination to ensure that it is of good quality.

Fungal propagation

Aspergillus oryzae was cultured in Potato Dextrose Agar (PDA). Eight ml of media was poured into a sterile test tube and sterilized at 121°C for 15 min. Slant cultures were then inoculated and incubated at 27°C for 4 days. These were maintained in a refrigerator (7°C) after the incubation period and subcultured weekly.

Fungal spore estimation and preparation

The diluent was prepared by adding 0.05 g Tween 80 (Batch No. B-891) to100 ml distilled water. A diluent of 10 ml were added to a 4-day old slant. This was then shaken and scraped with a sterile wire loop to make a spore suspension. The spore suspension was then diluted to 10^{-1}. The spore count in the suspension was estimated using

Neubauer haemocytometer (0.100 mm deep). To estimate the quantity of fungal spores, haemocytometer was prepared by cleaning all the surfaces and cover slip carefully. These were then dried completely by using a non-linting tissue. The cover slip was then centered on the haemocytometer. Taking into consideration the cleanliness of the pipette as well as thorough mixing of the cell suspension, avoiding injection of bubbles into chambers, and not overfilling or underfilling the chamber, a small drop of cell suspension was pipetted into one of the two counting chambers. The spores were then counted in each of the four 0.1 mm^3 corner squares. The spore count was determined by calculating the total spores counted in the four corners and was calculated using the equations:

$$\text{Spores/ml} = n \times df \times 10^4$$

where, n = the average cell count number per square of the four corner squares counted
df = dilution factor

$$\text{volume of estimated spore suspension} = (A \times V) / B$$

where, A = required spore concentration
V = volume of spore suspension needed
B = value of estimated spore suspension

Total volume of spore suspension = vol. of estimated spore suspension + vol. of distilled water

Fungal lactase production

Lactose-hydrolyzed milk was produced according to the procedures of Rosal (2012). Fungal spore suspension was standardized to 1.12 x 10^7 spores/ml. Ten ml of this suspension were then put into 100 ml buffalo's milk and incubated at 27°C for 4 hr to make it produce lactose-hydrolyzed milk as was done in the study of Rosal (2012). In this study, the milk samples were incubated at 30°C. Prior to any treatment, the pH was determined. The incubated milk was then centrifuged (6000 rpm for 20 min) at refrigerated temperature (4°C) and supernatant was saved. The supernatant was then added to another set of whole buffalo's milk and incubated for another 4 hr. Ten ml of supernatant were added to another 100 ml of whole buffalo's milk.

Sample collection

Milk samples were taken from each treatment before and after incubation. The samples were immediately stored at 4°C and analyzed for lactose and reducing sugar contents.

Lactose content determination

Lactose contents of the samples were determined using the Picric Acid Method (Dehn and Hartman, 2010). Standard solutions were prepared by transferring 0.10, 0.30, 0.5 up to 1.50 ml standard lactose solution into 30-ml test tube. One ml sodium carbonate solution was then added and the mixture was heated to boiling water for 30 min. The tubes were then cooled to room temperature and diluted to 20 ml with distilled water. These were then mixed by inverting the tubes. A portion of the resulting solution was then

transferred to a colorimeter tube and absorbance was read at 520 nm. Blank containing 2 ml picric acid solution and 1 ml sodium carbonate solution was also run at the same time the samples prepared. To prepare the samples, 0.10 g of milk was weighed into 125 ml Erlenmeyer flask. Ten ml saturated picric acid solution were then added. The solution was then shaken and stood overnight. The solution was then filtered into another flask using ordinary filter paper. A filtrate (0.2 ml) was then transferred into 30 ml test tube (0.05 ml for pure lactose) and processed just like the standards. The lactose content was calculated using the formula:

% lactose = ([S] x V x df x 100) / W

Where: = concentration of sample from standard curve, mg/ml
V = total volume of the sample solution, ml
df = dilution factor
W = weight of sample in mg

% RSD= (SD between replicates / X) x 100

Where: SD = standard deviation between replicates
X = mean

% Recovery = (actual concentration of QA samples / (theoretical concentration of the QA samples) x 100

Reducing sugar content determination

Prior to this reducing sugar test, the sugars of sample were extracted using the standard methods according to AOAC (1995). One ml of sample was obtained and added with 5 ml of absolute ethanol. The solution was mixed well and centrifuged at 3500 rpm for 10 min. The residue was saved while the supernatant was collected and put in a 50 ml volumetric flask. The residue was washed with 5 ml 80% ethanol, mixed and centrifuged at 3500 rpm for 10 min. The supernatant was then put in the flask where the first supernatant was contained. Washing was done twice. The supernatant collected was then diluted to 50 ml.

Reducing sugar content of milk was determined by DNS Method according to Miller (1959). To prepare the DNS reagent, 1 g dinitrosalicylic acid, 0.2 g phenol, 1 g sodium hydroxide and 20 g Rochelle salt (Potassium Sodium Tartrate) were dissolved in distilled water and diluted up to 100 ml in a volumetric flask. Sodium bisulfite (0.05 g) was added to the solution before use. Glucose solution was used as standard and was prepared by dissolving 100 mg dried glucose in 100 ml distilled water. During the DNS reducing sugar analysis, 1.5 ml aliquot of sugar extract from 50 ml extracted sugars was put in a test tube and was added with 1.5 ml DNS reagent. The solution was mixed and covered with glass marbles. This was then heated in boiling water bath for 15 min. After heating, this was cooled and diluted to 10 ml with distilled water and mixed thoroughly. The absorbance was then read at 550 nm using UV-Vis spectrophotometer. To calibrate the readings, calibration curve was prepared. Standard solutions of glucose containing 0.02 to 0.1 mg/ml were prepared. One ml of each solution was then taken and allowed to develop color following the same procedure as the samples. Absorbance readings were

then plotted against the concentration of glucose and the slope was calculated as mg glucose/ absorbance unit.

Statistical analysis

The data on the lactose and reducing sugar contents were subjected to Box plot determination, Shapiro-Wilk test and determination of outliers using Real Statistics Data Analysis Tools: Descriptive Statistics and Normality in Microsoft Excel 2007. The data on lactose content were not normally distributed, hence, were subjected to logarithmic transformation. The data on reducing sugar content were not transformed since distribution was normal. All data were subjected to Paired t-test: paired two sample for mean using Real Statistics Data Analysis Tools in Microsoft Excel 2007.

RESULTS AND DISCUSSION

The average lactose content of the whole buffalo's milk (T1) was 4.88% while the reducing sugar was 41.07±2.78 mg/ml (Table). The mean lactose content agrees with the values reported by Soliman (2005) and Enb *et al.* as cited by Rosal (2012), which ranges from 4.25% up to 5.35%. The milk used in the experiment had a pH of 6.35.

Lactose content

The lactose content showed a consistent decrease after inoculation with *A. oryzae* spores (T2) and after addition with supernatant from a previously *A. oryzae*-inoculated milk. The mean lactose contents (%) of the milk samples were significantly different among treatments with the lowest lactose content obtained from addition of supernatant (T3). Inoculation with *A. oryzae* resulted in 29.71% reduction in lactose content of the buffalo's milk. A higher reduction rate (48.77%) was attained after the whole milk was added with supernatant (T3).

Althaf *et al*. (2012) reported that *Aspergillus oryzae* utilizes several carbon sources for lactase production during submerged fermentation. Of the several carbon sources, lactose is second to glucose as the most utilized sugar. Usually, fungi hydrolyzes first the oligosaccharides into simpler sugars before they are utilized by the microorganisms,

Table. Mean lactose and reducing sugar contents of buffalo's milk treated with different inocula.

Treatment	Lactose content, %	Reducing sugars, mg/ml
Whole buffalo's milk (control)	4.88±0.25^{a}	41.07±2.78^{b}
Whole buffalo's milk inoculated with *A. oryzae*	3.43±1.15^{b}	35.24±2.93^{c}
Whole buffalo's milk added with supernatant	2.5±1.23^{c}	48.26±5.68^{a}

Means within column having different superscripts are significantly different (P≤0.05).
P-values of paired tests:
Lactose content: PT1-T2 = 0.01991; PT1-T3 = 0.00194; PT2-T3 = 0.01166
Reducing sugar content: PT1-T2 = 0.02443; PT1-T3 = 0.00408; PT2-T3 = 0.00627

but a few fungi, however, are reported to utilize some oligosaccharides directly (Bansal and Grover, 1970). *Aspergillus flavus,* a fungus similar in physiological and genetic traits with *A. oryzae* had poor growth and sporulation in basal medium with lactose as major carbon source as observed by Jorgensen as cited by Chang and Ehrlich (2010). His study revealed that lactose was not hydrolyzed by the fungus during its growth. In the present study, reduction in lactose content in milk inoculated with *A. oryzae* could be attributed to the utilization of lactose as a source of carbon for the production of lactase. The greater reduction in lactose content of milk with supernatant could be due to the presence of lactase that directly hydrolyzed the lactose in buffalo's milk. Lactase, being an extracellular enzyme, was easily extracted by centrifugation. The lactase was presumed to be present in the supernatant that was added to the milk in T3.

Reducing sugars

The reducing sugar content differed significantly among treatments. A reduction rate of 14. 20% was observed in whole buffalo's milk inoculated with *A. oryzae*. On the other hand, an increase of 17.51 % was observed in milk added with supernatant from a previously *A. oryzae*-inoculated milk.

Lactose itself exists as a reducing sugar; thus, the milk in the control (T1) had a reducing sugar content of 41.07%. During lactose hydrolysis, lactase split it into glucose and galactose which are both reducing sugars. Lactase hydrolyzes lactose by breaking the β-1, 4-glycosidic linkage which joins galactose to glucose (Berg *et al*., 2002). The decrease in reducing sugar content in T2 milk indicates that there was indeed no lactose hydrolysis that occurred during incubation. In addition, there was no lactase present yet in the milk at that time to breakdown the lactose. The lactose was utilized as a carbon source for lactase production.

It is apparent that lactase was indeed present in the supernatant added to buffalo's milk in T3. The increase in reducing sugar content was due to the release of glucose and galactose during lactose hydrolysis. The lower reduction rate in the lactose content of the milk in the present study compared with the study of Rosal (2012) implies that a longer incubation period is needed to facilitate lactose breakdown.

***Aspergillus oryzae* overall activity in buffalo's milk**

In a study done by Rosal (2012), direct incorporation of *A. oryzae* spores into the milk had 100% lactose reduction. A 100 ml whole buffalo's milk was directly inoculated with 10 ml spore suspension (1.12 x 10^7 spores/ml) and incubated within 4 hr at 27°C. It was then presumed that lactase was present as the fungal spores were suspended in the milk. However, the results of the current study did not agree with his results. This study still had high lactose content after incubation.

The current study adopted the submerged fermentation process since milk, a free-flowing substance, was used for lactase production. Submerged fermentation is best suited to bacteria and other microorganisms requiring high moisture. Althaf *et al*. (2012) reported that maximum lactase production by submerged fermentation of *Aspergillus oryzae* was observed at initial pH 5, temperature of 30°C, glucose and sodium citrate as carbon and nitrogen sources, for 6 days. The low reduction in lactose content that was observed in the current study could be due to the short incubation period that was used. Furthermore, the fungal enzymes are found to be stable over a broad range of pH (Zagustina *et al*., 1975) while their optimal temperature is 55°C (Agrawal *et al.,* 1989). In this study, fungal lactase was produced at a relatively higher pH than in the study of Althaf *et al*. (2012) and

the temperature was within the range of 30-55°C. With these related studies regarding the conditions for optimum lactase production and action on certain substrate, it can be concluded that the broader considerations on temperature, pH and incubation periods are required.

An additional option for lactase production is the use of solid state fermentation. Solid state fermentation offers great possibilities when these are used (Bhargav *et al.*, 2008). Fungi like *A. oryzae* are different from any other microorganisms as they usually grow in solid substrates. The substrate itself acts as a carbon source and enzyme production occurs in the absence or near absence of free water.

CONCLUSION

Reduction in lactose content with direct inoculation of *Aspergillus oryzae* is due to utilization of the milk sugar for microbial activity and lactase production. Lactase could be obtained from the supernatant of *A. oryzae* inoculated milk and could be used as inoculum to reduce lactose. An incubation period longer than 4 hr could be used to increase percent reduction in lactose content of buffalo's milk. A study on the enzyme stability of lactase and the optimum conditions for lactase production would help determine the commercial potential of *Aspergillus oryzae* in the dairy industry.

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