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EFFECTIVE CONDITIONS OF MOISTURE CONTENT, INOCULUM RATE, AND MEDIUM COMPOSITION FOR THE PRODUCTION OF CELLULASE, AMYLASE, AND XYLANASE BY FERMENTATION OF SLAUGHTERHOUSE RUMEN CONTENTS

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ABSTRACT

This study was conducted to investigate the optimum conditions of moisture, inoculum, and medium ingredients, for enzyme production by solid-state fermentation of rumen content, a slaughterhouse by-product, intended to be used as a feed additive. *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Saccharomyces cerevisiae* were used as starter cultures. Slaughterhouse rumen content, molasses, cornstarch, soybean meal, wheat bran, and ammonium chloride were selected as the variables of the medium ingredients. Moisture content and inoculum were used as the variables of the fermentation condition. The enzyme activities of cellulase, amylase, and xylanase were assessed using the Taguchi experimental design. Thus, the study was conducted with 18 experimental conditions defined by various combinations of 8 variables. To determine the integrated effects of variables on all 3 enzymes simultaneously, the standardized value of signal-noise (SN) ratio was calculated, following which, the average value of the standardized SN ratios was used to determine the effects of different levels of variables on enzyme activity. In conclusion, the moisture content, molasses, and ammonium chloride were detected as effective variables with significance ($p < 0.05$) for the improvement of enzyme production during the fermentation of slaughterhouse rumen content. We found that 40% moisture, 20% rumen contents, 1% inoculum, 10% molasses, 2% cornstarch, 5% soybean meal, 30% wheat bran, and 0.1% ammonium chloride of total fermentation medium were found to be the effective levels of variables for the simultaneous production of the three enzymes that were assessed. This is the first study to report the development of the feed additive containing various enzymes produced by recycling of rumen contents, a slaughterhouse by-product.

Key words: Rumen contents, Fermentation, Cellulase, Amylase, Xylanase, Taguchi method.

INTRODUCTION

Recently, rumen contents, obtained as a by-product from slaughterhouses, have become available in Korea as a feed or feed additive resource, since it contains various unknown growth factors, which may promote the growth of rumen microorganisms and improve rumen function (Kim *et al.* 2000). In fact, it has been reported that there were no negative effects on feed intake and body weight gain when dried slaughterhouse rumen contents (up to 4%) were added to the feeds for lambs (Salinas-Chavira *et al.* 2007). Dried slaughterhouse rumen contents can replace up to 30% of the roughage in the diet of beef cattle (Rincon *et al.* 2010) and 37.5% of the corn in chicken feed (Adeniji and Jimoh 2007). In a study by Khattak (2009), silage produced by 60 days fermentation of slaughterhouse rumen contents with wheat straw and molasses can partially substitute total mixed ration (TMR) in buffalo feed. Slaughterhouse rumen content has also been utilized as an ingredient in the medium for the fermentation of *Aspergillus oryzae*, a direct-fed microbial (Kim *et al.* 2000).

Fermentation for the production of feed additives is generally aimed for maximizing bacterial growth or enzyme production. Supplementing with enzymes such as cellulase, amylase, and xylanase can improve the efficiency of nutrient degradation and the productivity of livestock, especially in ruminants that can utilize cellulosic materials as an energy source (Eun *et al*. 2007; Klingerman *et al*., 2009).

For efficient fermentation, identification of optimum medium ingredients and fermentation conditions is very important. In previous studies, several statistical methods have been applied to evaluate fermentation efficiency (Cho *et al*., 2010). For example, the Taguchi method is known to be a high-throughput statistical method based on fractional factorial design (Lee *et al*., 2010). When calculating responses employing the Taguchi experimental design, three approaches, namely, "normal-is-better," "smaller-is-better," "larger-is-better," can be applied according to the purpose of the experiment. Responses are then transformed into signal-noise (SN) ratios, which are more representative of effect patterns, allowing effective targeting of the experiment

with reduced dispersion of responses (Lee *et al.* 2010, Lim and Yang 2006). Generally, bacterial cell growth, antioxidant activity, and enzyme activity have been used as indicators for the optimization of fermentation conditions. When there is more than one target, appropriate integration of various responses should be applied, and standardization, using normal standard distribution, has been widely used (Lee *et al.* 2010).

The present study was conducted to develop slaughterhouse rumen content as feed additives by intensifying the enzyme activities of cellulase, amylase, and xylanase through the process of fermentation. To improve the enzyme activities, we identified the effective conditions of moisture content, inoculum, and medium ingredients (including slaughterhouse rumen content) by using a statistical method.

MATERIALS AND METHODS

Bacterial strains and culture media: *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Saccharomyces cerevisiae* were used as starter cultures. To prepare starter cultures, the two bacilli strains and *S. cerevisiae* were cultivated in Luria Broth (LB, Difco, USA) and yeast-malt extract (YM, Difco, USA) broth media, respectively. Seed cultures were prepared using test tubes with 10 mL of working volume and incubated at 30°C for 20 h with agitation (100 rpm).

Screening of medium components: A total of 8 factors, including slaughterhouse rumen contents, were employed to evaluate their effects on enzyme production under solid-state fermentation conditions. Table 1 lists the medium components used and their concentrations. Using the Taguchi DOE (Design of Experiment), three levels of each variable were orthogonally arranged and 18 different experimental runs were produced (Table 2). The SN ratios of cellulase, amylase, and xylanase activities under the various tested conditions in the 18 experimental runs were calculated using the 'Taguchi larger-is-better' approach. The effect of each medium component was estimated using an average SN ratio. To evaluate the impact of the variables on cellulase, amylase, and xylanase activities simultaneously, SN ratio assigned to each enzyme activity was standardized. Prior to standardization, a normal distribution test of SN ratios was performed using the Anderson-Darling method (Anderson and Darling 1952). The calculation procedures for SN ratios, standardization, and analysis of average were done as shown below.

Calculation of SN ratio: SN ratio was calculated using the observed cellulase, amylase, and xylanase activities from 18 experimental runs by using the following equation:

$$SN_R^E = -10\log(\frac{1}{r}\sum_{i=1}^{r}\frac{1}{(y_i^R)^2})$$

SN_R^E represents the SN ratio of E enzyme activity at the R^{th} experimental run (R = 1,2,·,18), y_i^R is the observed E enzyme activity from the R^{th} experimental run, and i represents replicates (i = 1,2,··r).

Standardization: To determine the effective levels at which the variables affect the three enzymes simultaneously, standardization of the different SN ratios, obtained from the activities of each individual enzyme, was employed. SN ratio values from the 18 experimental runs were used as the original values. New Z values were calculated using the average and standard deviation with the following equation:

$$Z_R^E = \frac{(SN_R^E - «_E)}{±_E} \sim N(0,1)$$

Z_R^E is the standardized SN ratio (Z value) of E enzyme activity and R^{th} experimental run, and $«_E$ and $±_E$ are the average and standard deviation of the SN ratio, respectively.

Average value: The simultaneous effects of variables on all three enzymes activities were evaluated by averaging the standardized SN ratios ($\overline{EX}$). The $\overline{EX}$ value was calculated using the following equation:

$$\overline{EX}_{a,j} = \frac{\sum Z_R^E}{n_j \times 3}, (R \in X_{a,j})$$

$\overline{EX}_{a,j}$ is the average of standardized SN ratios at the j^{th} level of X_a medium component and n_j represents the number of j^{th} levels assigned to medium components.

Culture conditions: The medium for solid-state fermentation (SSF) assigned to each experimental run was determined according to the DOE in Table 2. The total medium weight of each run was 200 g, and the medium was placed in an aluminum box. SSF was performed at 30°C for 48 h. The medium was mixed once after 24 h incubation by using a sterilized glass stick. Each run of SSF was repeated in triplicate.

Analyses of enzyme activity: Cellulase, amylase, and xylanase activities were analyzed according to the methods of Son *et al.* (2006), but with minor modifications. Briefly, the procedures used were as follows. For the analysis of cellulase, amylase, and xylanase, the substrates used were carboxymethyl cellulose (CMC; Sigma), soluble starch (Difco), and birchwood xylan (Sigma), respectively. Substrate solution was prepared using 1% substrate dissolved in 10 mM Tris-HCl (pH 7.0), except CMC (0.5%). Enzyme reactions were started by mixing 1 g of sample with 9 mL

of substrate solution and the reaction was maintained at 37°C for 1 h with agitation (150 rpm). After the reaction, 0.3 mL of the reaction mixture supernatant was centrifuged (10,000 rpm, 5 min) and 0.3 mL of DNS solution (3, 5-dinitrosalicylic acid, 1%; sodium sulfate, 0.05%; sodium hydroxide, 1%) was added to the supernatant. Thereafter, the mixture was heated at 90°C for 10 min. After cooling, 50 µL of 40% potassium sodium tartrate was added to the mixture, and precipitated particles were removed via filtration (pore size, 0.45 µm). The optical density of the solution was then detected at 575 nm using a spectrophotometer (Shimazu, Japan). The optical density of the SSF medium was regarded as background and was excluded by measuring the optical density of a blank solution prepared with the sample and 10 mM Tris-HCl without substrate (CMC, soluble starch and birchwood xylan). The amount of sugars released by enzyme reactions was estimated by regression analysis of a standard curve that was generated with known concentrations of glucose or xylose. Glucose was used as the standard for cellulase and amylase activities, while for xylanase, xylose was used as the standard. One unit of enzyme activity was defined as 1 µM sugar (glucose or xylose) from 1 g of sample per 1 min.

Statistical analysis: For the construction of Taguchi DOE, calculation of SN ratios, analysis of variance (ANOVA), and normal distribution tests were performed using the MINITAB® statistical software (version 14.0, Minitab Inc., USA). Multiple comparisons of average SN ratios assigned to each enzyme activity were performed using a general linear model with Duncan's multiple range test by statistical program R (version 2.14.2).

RESULTS AND DISCUSSION

Effects of medium components on enzyme activities: Moisture, rumen content, inoculum, molasses, cornstarch, soybean meal powder, wheat bran, and ammonium chloride were used as variable components of the culture conditions. Different levels of each variable were assigned to 18 different experimental runs, and the enzyme activities from each of these runs are summarized in Table 2. The average viable bacterial cell counts from all experimental runs were $3.5 \pm 2.3 \times 10^9$ CFU/g, indicating that the microbial growth during SSF was normal. Cellulase activity detected from each experimental run ranged from 22.7 U to 93.1 U, with an average value of 49.5 U. Amylase activity was greatly influenced by the variables, as evidenced by the large differences in its activity (6.1–240.3 U) under different fermentation conditions. The average amylase activity was 68.9 U. Xylanase activity was relatively low, when compared with the activities of the other enzymes. Minimal and maximal xylanase activities were 0.2 U and 6.8 U, respectively, with an average of 3.2 U. SN ratios for cellulase, amylase, and xylanase were calculated using larger-the-better characteristics. Their average values arranged to experimental runs were 32.3, 30.6, and 6.8 for cellulase, amylase, and xylanase, respectively, as shown in Table 2.

Effect on cellulase activity: The effects of variables at different levels on cellulase activity are summarized in Table 3. Significant differences of average SN ratio among levels of variables were found in both of molasses and ammonium chloride ($p < 0.05$). When moisture content was increased from 30% (level 1) to 40% (level 2), cellulase activity was slightly increased. For rumen content, the highest average SN ratio was found at 20% (level 2). The average SN ratio of cellulase activity was increased in direct proportion to the inoculum. The SN ratio for various quantities of molasses tested and those from 5% (level 2) and 10% (level 3) of molasses levels were higher than that of 1% of molasses (level 1), significantly ($p < 0.05$). However, there was no significant difference between 5% and 10% addition levels ($p > 0.05$). The average SN ratio of cellulase activity decreased with increasing levels of starch. Second level (2.0%) of soybean meal showed the highest average SN ratio, which was higher than both of 1.0% (level 2) and 5% (level 3) addition levels of soybean meal. The assessment of wheat bran addition showed average SN ratio at 50% (level 3), which was higher than that of 15% (level 1) and 30% (level 2). Increasing ammonium chloride level significantly decreased ($p < 0.05$) cellulase activity. Thus, molasses and ammonium chloride were detected as the important medium components that can significantly ($p < 0.05$) affect cellulase activity in fermentation products made from slaughterhouse rumen content. In summary, cellulase activity was directly proportional to the quantity of molasses used and inversely proportional to the ammonium chloride quantity.

Effect on amylase: Among the variables that influenced amylase activity in fermentation products that used slaughterhouse rumen content, ammonium chloride was detected as a significant medium component (Table 3). In the case of moisture content, the average SN ratio showed a slight decrease, with increasing levels of moisture. With respect to rumen content, the average SN ratio of level 1 was higher than that of level 2, but lower than that of level 3. The first level of inoculum had the highest average SN ratio and the second level 2 of molasses addition yielded the highest SN ratio. There were no dramatic changes in the SN ratio, which was related to the levels of cornstarch or soybean meal. In the case of wheat bran, the highest average SN ratios were found at level 1. As with cellulase activity, amylase activity was also significantly decreased by increasing ammonium chloride level ($p < 0.05$). Thus, ammonium chloride was found to be an important medium component, which can

significantly ($p < 0.05$) affect amylase activity.

Effect on xylanase: None of the variables that were analyzed in this study affected xylanase production (Table 3). The average SN ratios increased in direct proportion to the increasing levels of moisture. In the case of rumen contents, the highest SN ratio was observed at level 2. The SN ratio at level 2 of the inoculum rate was higher than both of level 1 and level 3. With respect to molasses, level 3 yielded the highest SN ratio. The highest SN ratios were found at levels 2 and 3 for cornstarch and soybean meal, respectively. Higher SN ratios were observed at levels 2 and 1 than at level 3 for wheat bran and ammonium chloride, respectively.

Average values of standardized SN ratios: To determine the effects of variables on the three enzymes simultaneously, the SN ratios calculated from each enzyme activity were standardized using a standard normal distribution. However, after standardization, SN ratio calculation was not analyzed by larger-the-best characteristics, because standardized SN ratios with negative values can be converted to positive values during the calculation, which in turn may result in the overestimation of values and a lack of precision. Thus, only average values were used at the final step for calculating simultaneous effects. Prior to standardization, the normality of the distribution of SN ratios for each enzyme described in Table 2 was tested and all enzymes showed normality as follows: cellulase ($p = 0.846$); amylase ($p = 0.083$); and xylanase ($p = 0.161$). The responses and SN ratios from each enzyme were then standardized (Table 4). By analysis of variance, the

Table 1. Variables and their actual values assigned to each coded level

Variables		No. of levels	Actual values of variables assigned to levels, % (w/w)		
			1	2	3
X_1	Moisture content [1)]	2	30.0	40.0	-
X_2	Rumen content	3	0.0	20.0	30.0
X_3	Inoculum	3	0.1	1.0	5.0
X_4	Molasses	3	1.0	5.0	10.0
X_5	Corn starch	3	1.0	2.0	5.0
X_6	Soybean meal	3	1.0	2.0	5.0
X_7	Wheat bran	3	15.0	30.0	50.0
X_8	NH_4Cl	3	0.1	1.0	2.0

[1)] Only two different levels of moisture content were employed in this experiment.

Table 2. Experimental design matrix (L-18 orthogonal array) and observed enzyme activities

Runs	Variables[1)] and their levels								Enzyme activities, U/g			SN ratio		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Cellulase	Amylase	Xylanase	Cellulase	Amylase	Xylanase
1	1	1	1	1	1	1	1	1	36.6 ±13.1[2)]	121.5 ± 3.4	2.1 ± 0.5	29.9	41.7	5.5
2	1	1	2	2	2	2	2	2	41.0 ± 2.4	24.7 ± 4.2	4.2 ± 0.4	32.2	27.4	12.3
3	1	1	3	3	3	3	3	3	34.2 ± 1.4	6.1 ± 0.8	1.6 ± 0.1	30.7	15.5	3.9
4	1	2	1	1	2	2	3	3	22.7 ± 4.0	6.5 ± 1.0	0.9 ± 0.2	26.8	16.0	-1.3
5	1	2	2	2	3	3	1	1	50.2 ± 5.3	240.3 ± 7.1	6.8 ± 0.7	33.9	47.6	16.5
6	1	2	3	3	1	1	2	2	59.2 ± 5.9	16.1 ± 7.1	1.5 ± 0.2	35.3	22.0	3.0
7	1	3	1	2	1	3	2	3	36.4 ±10.6	18.6 ± 2.0	1.8 ± 0.7	30.1	25.2	1.5
8	1	3	2	3	2	1	3	1	93.1 ± 3.4	159.7 ± 6.8	3.1 ± 0.4	39.4	44.0	9.5
9	1	3	3	1	3	2	1	2	34.4 ± 5.7	24.7 ± 3.9	0.2 ± 0.1	30.4	27.6	-15.3
10	2	1	1	3	3	2	2	1	66.7 ± 2.6	169.4 ± 3.4	5.3 ± 1.0	36.5	44.6	13.9
11	2	1	2	1	1	3	3	2	49.8 ± 8.5	7.6 ± 2.0	1.7 ± 0.9	33.5	16.7	-3.4
12	2	1	3	2	2	1	1	3	37.5 ± 2.3	15.3 ± 2.0	1.1 ± 0.1	31.5	23.4	0.5
13	2	2	1	2	3	1	3	2	48.6 ± 4.1	50.0 ± 3.4	6.2 ± 1.3	33.7	33.9	15.1
14	2	2	2	3	1	2	1	3	63.9 ± 5.2	23.6 ± 2.0	3.5 ± 0.4	36.0	27.4	10.7
15	2	2	3	1	2	3	2	1	67.4 ± 1.7	157.6 ± 3.9	6.8 ± 0.1	36.6	44.0	16.6
16	2	3	1	3	2	3	1	2	45.8 ± 3.4	47.2 ± 2.0	3.6 ± 0.3	33.2	33.5	11.0
17	2	3	2	1	3	1	2	3	31.0 ±12.0	6.8 ± 1.1	3.9 ± 0.8	25.3	16.3	11.4
18	2	3	3	2	1	2	3	1	72.6 ± 6.2	145.1 ± 3.4	3.7 ± 0.7	37.1	43.2	10.8
Mean±SD									49.5 ±17.4	68.9 ±72.5	3.2 ± 2.0	32.3 ± 3.6	30.6±11.0	6.8±8.1

[1)] Variables: X_1, moisture content; X_2, rumen content; X_3, inoculum; X_4, molasses; X_5, corn starch; X_6, soybean meal; X_7, wheat bran; X_8, NH_4Cl.
[2)] Mean ± standard deviation for triplicate experiments.

Table 3. Average SN ratios from the effects of variables on individual enzyme activities

Variables	Cellulase			Amylase			Xylanase		
Levels:[1)]	1	2	3	1	2	3	1	2	3
Moisture content	32.08±1.23[2)]	33.71±1.23	-	29.67±3.99	31.44±3.74	-	3.96±3.04	9.62±2.23	-
Rumen content	32.38±0.97	33.72±1.46	32.58±2.09	28.22±5.06	31.82±5.06	31.63±4.41	5.45±2.73	10.10±3.10	4.82±4.30
Inoculum	31.70±1.40	33.38±1.92	33.60±1.25	32.48±4.32	29.90±5.43	29.28±4.79	7.62±2.76	9.50±2.76	3.25±4.42
Molasses	30.42±1.71^{b}	33.08±0.99^{ab}	35.18±1.24^{a}	27.05±5.31	33.45±4.09	31.17±4.81	2.25±4.66	9.45±2.79	8.67±1.76
Corn starch	33.65±1.25	33.28±1.78	31.75±1.59	29.37±4.39	31.38±4.62	30.92±5.59	4.68±2.26	8.10±2.87	7.58±4.93
Soybean meal	32.52±1.97	33.17±1.67	33.00±0.96	30.22±4.63	31.03±4.46	30.42±5.56	7.50±2.24	5.18±4.66	7.68±3.38
Wheat bran	32.48±0.95	32.67±1.81	33.53±1.83	33.53±3.83	29.92±4.79	28.22±5.63	4.82±4.60	9.78±2.50	5.77±2.97
NH_4Cl	35.57±1.37^{a}	33.05±0.67^{ab}	30.07±1.54^{b}	44.18±0.79^{a}	26.85±2.72^{b}	20.63±2.17^{b}	12.13±1.78	3.78±4.73	4.45±2.20

[1)]Conditions for each level assigned to variables: Moisture content, 30% (1), 40% (2); rumen content, 0% (1), 20% (2), 30% (3); inoculum, 0.1% (1), 1.0% (2), 5.0% (3); molasses, 1% (1), 5%(2), 10% (3); corn starch, 1% (1), 2% (2), 5% (3); soybean meal, 1% (1), 2% (2), 5% (3); wheat bran, 15% (1), 30% (2), 50% (3); NH_4Cl, 0.1% (1), 1.0% (2), 2.0% (3).
[2)]Mean ± standard error
Different superscripts in same enzyme activity assigned to each variable mean significantly different ($p < 0.05$).

Table 4. Standardized SN ratio of each enzyme activity

Z_R^E	Z^C	Z^A	Z^X
Z_1	-0.83	1.01	-0.16
Z_2	-0.19	-0.29	0.68
Z_3	-0.63	-1.37	-0.36
Z_4	-1.71	-1.33	-1.00
Z_5	0.27	1.55	1.21
Z_6	0.68	-0.78	-0.46
Z_7	-0.78	-0.48	-0.66
Z_8	1.81	1.23	0.33
Z_9	-0.70	-0.27	-2.74
Z_{10}	1.00	1.28	0.89
Z_{11}	0.18	-1.26	-1.27
Z_{12}	-0.39	-0.65	-0.78
Z_{13}	0.23	0.31	1.04
Z_{14}	0.88	-0.29	0.48
Z_{15}	1.03	1.22	1.22
Z_{16}	0.07	0.27	0.53
Z_{17}	-2.13	-1.30	0.57
Z_{18}	1.19	1.16	0.50

In Z_R^E, E and R represent enzyme and number of experimental runs, respectively.
Z^C, Z^A, and Z^X represent standardized SN ratios of cellulase, amylase, and xylanase, respectively.

moisture content, molasses, and ammonium chloride were found to significantly influence on enzyme production from solid-state fermentation (Table 5). The results obtained using the average values of standardized SN ratios ($\overline{EX}_{a,j}$) for estimating the simultaneous effects of variables on the three enzymes are shown in Figure 1. In conclusion, molasses and ammonium chloride were detected as significantly effective medium ingredients ($p < 0.05$) and moisture content as effective fermentation condition with significance ($p < 0.05$) for the improvement of enzyme production during the fermentation of slaughterhouse rumen content (Table 5). The effective levels of moisture, molasses, and ammonium chloride were determined to be 40%, 10% and 0.1%, respectively. Using this approach, the fermented rumen contents can be used to provide enzymes such as cellulase, amylase, and xylanase along with various nutrients in the animal feed. In ruminant diets, supplementing enzymes such as cellulase, amylase, and xylanase, may improve the efficiency of nutrient

utilization and livestock productivity. Furthermore, it has been previously reported that supplementing cellulase increases cellulose utilization rate, microbial protein synthesis, and protein degradation rate in the rumen (Eun *et al.* 2007, Kung *et al.* 2002). Adding amylase to the diet improves starch degradation rate and it has also been reported that amylase increases milk production in dairy cattle and daily weight gain in beef cattle by improving starch utilization from feeds (Klingerman *et al.* 2009, Tricarico *et al.* 2007). This study demonstrates that slaughterhouse rumen contents can be recycled as a value feed additive.

Table 5. Analysis of variance[(1)] for standardized SN ratios

Items	DF	SS	MS	*F* value	*P* value
Moisture content (X_1)	1	2.6315	2.6315	5.56	0.024
Rumen content (X_2)	2	1.7951	0.8975	1.90	0.164
Inoculum (X_3)	2	0.6090	0.3045	0.64	0.531
Molasses (X_4)	2	7.5389	3.7695	7.97	0.001
Corn starch (X_5)	2	0.3529	0.1765	0.37	0.691
Soybean meal (X_6)	2	0.0466	0.0233	0.05	0.952
Wheat bran (X_7)	2	0.1907	0.0953	0.20	0.818
NH_4Cl (X_8)	2	22.8615	11.4308	24.17	<0.001
Error	38	17.9738	0.4730		
Total	53	54.0000			

[1)] DF, SS, and MS represent degrees of freedom, sum of squares, and mean of squares, respectively.

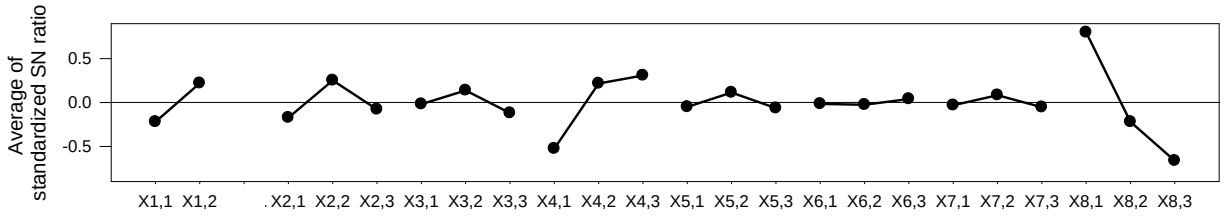

Figure 1. Average SN ratios of enzyme activity and average of means from each level of variables.
The average of SN ratios calculated with standardized cellulase and xylanase activities are represented. In the Xa,j of the x-axis, a and j represent variables and their levels are described in Table 1 (X1, X2, X3, X4, X5, X6, X7 and X8 are moisture content, rumen content, inoculum, molasses, corn starch, soybean meal, wheat bran and ammonium chloride, respectively).

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