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The Use of Fungal Inoculants in the Ensiling of Potato Pulp: Effect of Temperature and Duration of Storage on Silage Fermentation Characteristics

Okine, A.*, Y. Aibibula[1], M. Hanada and M. Okamoto

Department of Agro-environmental Science. Obihiro University of Agriculture and Veterinary Medicine Obihiro Hokkaido. 080-8555 Japan

ABSTRACT : A 3×3 factorial design experiment was conducted to investigate the effect of temperature and duration of storage on the fermentation quality of potato pulp ensiled with two fungal inoculants under laboratory conditions. The inoculants, *Rhizopus oryzae* (R) and *Amylomyces rouxii* (A) were each added to potato pulp material to contain at least 1×10^6 CFU/g fresh matter, and silages without additives served as controls. The silages were stored under three temperature regimes; 4, 12 and 25°C. Three silos per treatment from every temperature regime were opened on days 7, 24 and 40 days after ensiling to investigate treatment effects on fermentation quality, starch and sugar concentrations. Increase in temperature and duration of storage had a positive significant effect ($p<0.01$) on the fermentation quality of potato pulp silage (PPS). The inoculants had little effect ($p>0.05$) on the fermentation quality of the silages. Sugar concentration in the silages decreased with increase in temperature ($p<0.01$) but increased ($p<0.05$) with progression of duration of storage. The fungal inoculants had no effect on starch degradation in PPS. The results suggest that storage temperature and duration of storage are more important in determining the rate of fermentation than addition of the fungal inoculants in PPS. (**Key Words :** Amylomyces Rouxii, Potato Pulp, Rhizopus Oryzae, Temperature, Duration of Storage)

INTRODUCTION

Ensiling of potato pulp, a by-product of the potato starch industry, is considered a viable method for its preservation and subsequent use as feed for ruminants due to its high nutritional value (Aibibula et al., 2004; Okine et al., 2005). Although the use of bacterial inoculants in the enhancement of fermentation quality of silages is well established (Gordon, 1989; Meeske et al., 1999; Guan et al., 2002), few studies (Oda et al., 2002; Okine et al., 2005) have however, investigated the use of fungal or aerobic inoculants in an anaerobic process such as ensiling. Potato pulp contains mainly starch, peptic substances and mineral salts (Mayer and Hillebrandt, 1997) which are susceptible to enzymatic breakdown during ensilage. The fungus *Rhizopus oryzae* contains the enzymes pectinase and lactose dehydrogenase which, used as an inoculant, could break down starch and pectin into sugar (Skory, 2000; Erdogan et al., 2001) thus increasing the substrate availability for lactic acid fermentation in potato pulp silage (PPS). This assertion is further strengthened by the fact that *R. oryzae* possesses fermentative enzymes that allow the fungus to grow in the absence of air (Skory, 1998) and can produce lactic acid in potato pulp even under airtight conditions (Oda et al., 2002). However, our (Okine et al., 2005) previous investigation on the use of *R. oryzae* in PPS fermentation did not confer any advantages over the silage without the inoculant in lactic acid production. Of the reasons considered for the lack of effect was the ambient temperature during the ensiling period. The success of microbial inoculants used to enhance ensiling fermentation is partly dependent on the temperature, since different inoculants have various temperature optima (McDonald et al., 1991). Hachmeister and Fung (1993) reported that temperature (between 27 and 35°C) and length of fermentation were crucial factors in *Rhizopus* fermentation of tempeh, a mold-modified indigenous fermented food made from soybeans or cereal grains in Indonesia. In temperate regions like Hokkaido, northern Japan, where

* Corresponding Author: Okine Abdul Razak. Tel: +81-155-49-5484, Fax: +81-155-49-5775, E-mail: razak69@hotmail.com

[1] Applied Greenstock Science, Faculty of Bioresources, Mie University, Mie, Japan.

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potato pulp is produced abundantly annually and preservation by ensiling is an option. temperature may be important in determining the efficacy of the fungus in PPS fermentation. The fungus *Amylomyces rouxii* is taxonomically identical to *R. oryzae* in characteristics and ability to produce lactic acid (Abe et al., 2004). Both were used in this study because of their ability to hydrolyze starch into glucose and their use as starters for potato pulp fermentation (Abe et al., 2003; Abe et al., 2004).

This study investigated the effect of various temperature regimes and duration of storage on the fermentation quality of potato pulp silage inoculated with the two fungi.

MATERIALS AND METHODS

Silage preparation

Fresh potato pulp, obtained from a local starch-processing factory in Hokkaido, northern Japan, in 2003, was used in this study. The two inoculants *Rhizopus oryzae* IFO 4707 (R) and *Amylomyces rouxii* CBS 438-76 (A), originally from freeze-dried cultures, were each grown on media containing malt extract agar for fungi and incubated at 25°C in Petri dishes 72 h prior to use. The fungi, including the media, were added separately to the potato pulp at the rate of 1.0% (w/w) in fresh matter, enough to produce at least 1×10^6 colony forming units (CFU) g^{-1} on use (manufacturer's statement). Three treatments made up of potato pulp without additive (control, C), R treated silage (PR) and A treated silage (PA) were prepared and ensiling was done in polyethylene silos (300 mm×200 mm×0.15 mm). Eighty-one silos were made; 27 for each of the three treatments. Each silo was filled with 500 grams of potato pulp and 5 grams fresh weight of the medium containing either R or A, were added to 54 silos and mixed thoroughly before filling. To ensure compaction, the silos were pressed gently and a vacuum air deflator was used to remove any air from the silos followed immediately by heat-sealing and further strengthening with adhesive tape. Twenty-seven silos from each of the three treatments were kept under constant temperature regimes of 4, 12 and 25±1°C, respectively. Pre-silage material was sampled on day 0 for immediate determination of pH and dry matter (DM) content. Three bags per treatment from every temperature regime were randomly selected and opened on days 7, 24 and 40 post-ensiling and sampled to investigate treatment effects on the fermentation quality through measurement of DM, pH, lactic acid, volatile fatty acids (VFA), sugar and starch contents of the resultant silages.

Chemical analyses

Potato pulp material and silages were dried for at least 24 h using a freeze-dryer and sub-samples were ground to pass a 1mm screen for subsequent analyses. Dry matter was determined from sub-samples by drying in a forced-draught oven at a constant temperature of 135°C for 2 h. The pH of pre-silage material and silages was determined from aqueous extracts using pH meter equipment (pH meter HM-30G, TOA Electronics Ltd, Tokyo, Japan); lactic acid was determined from aqueous extracts by the colorimetric method of Baker and Summerson (1961); sugar (net sugar, principally monosaccharides) was determined from dried samples by extraction with 80% ethanol at a stable temperature of 80°C and acid hydrolysis with anthrone solution (containing anthrone and thiourea in a solution of concentrated sulfuric acid). Starch was determined in the dried residue following sugar extraction, with 60% Perchloric acid ($HClO_4$) and continuous boiling for 2 h. The starch concentration was then determined by its color reaction for glucose at room temperature using a test kit (Glucose B Test Wako, Wako Pure Chemical Industries Ltd, Tokyo, Japan). The sugar and starch concentrations were measured using a spectrophotometer with a standard solution from the test kit (mentioned above) at peak transmissions of 650 mμ and 420 mμ, respectively. These methods are fully described by Abe (1988). The concentrations of acetic acid, a volatile fatty acid (VFA), were analyzed by gas–liquid chromatography (Shimadzu GC-14 A, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm i.d. ×30 m×3.0 μm) and using 2-ethyl-*n*-butyric acid as the internal standard as described by Nguyen et al. (2004).

Statistical analyses

Silage fermentation data were analyzed in a 3×3 factorial design using ANOVA in the general linear model procedure of SAS (1996). Main effects and interaction between treatments and contrasts were performed for responses of inoculation to temperature and duration of storage. Significant mean differences in effects were separated using the least significant difference test and p-values less than 0.05 considered statistically significant. In the regression equations, the measured parameters (pH and lactic acid) served as the dependent variables (Y) and storage temperature (°C) and duration of storage (days) as the independent variables.

RESULTS

Fermentation quality

The effects of inoculation, storage temperature and duration of storage and their interactions are given in Table 1. The pre-silage material contained 22.7% dry matter, 0.19, 0.13, 32.2 and 0.60% DM as lactic acid, acetic acid (the only VFA detected in PPS), starch and sugar, respectively, while values for the final (day 40) control silage at 25°C were 22.9% dry matter, and, respectively, 7.31, 1.07, 30.8

Table 1. Chemical characteristics of potato pulp material, main effects and interaction between inoculants, storage temperatures and duration of storage in fermentation characteristics of potato pulp silage after 40 days of ensilage (Values are least square means of three determinations given in % dry matter, unless otherwise stated)

	RM	Inoculants[1]			Temperature[2]			Duration of storage[3]				Significance[4]					
		C	PR	PA	T4	T12	T25	D7	D24	D40	Pooled SEM	I	T	D	I vs. T	I vs. D	T vs. D
DM (%)	22.7	21.4	21.1	21.3	21.2	21.2	21.3	21.4	21.5	21.0	0.36	NS	NS	NS	NS	NS	NS
pH	5.62	3.97	3.94	3.95	4.41^{a}	3.96^{b}	3.49^{c}	4.30^{a}	3.86^{b}	3.69^{c}	0.05	NS	***	***	NS	NS	***
Lactic acid	0.19	2.46	2.68	2.48	1.11^{b}	1.89^{b}	4.62^{a}	1.34^{b}	2.16^{b}	4.13^{a}	0.36	NS	***	***	NS	NS	***
Acetic eid	0.13	1.06	1.26	1.00	0.83^{b}	0.93^{b}	1.55^{a}	0.80^{b}	1.93^{a}	0.58^{b}	0.15	NS	**	***	*	**	NS
Starch	32.2	28.3	27.0	26.4	24.4	28.8	28.5	26.1	27.7	27.9	1.36	NS	NS	NS	NS	NS	NS
Sugar	0.60	0.80	0.94	0.82	0.88^{a}	1.16^{a}	0.53^{b}	0.57^{b}	1.01^{a}	0.97^{a}	0.11	NS	***	*	NS	NS	**

RM: pre-silage material; DM: dry matter; SEM: standard error of the means.

[1] C: silage without additive (control); PR: Rhizopus oryzae inoculated potato pulp silage; PA: Amylomyces rouxii inoculated potato pulp silage.

[2] T4, T12, T25, storage temperatures of 4, 12 and 25°C, respectively.

[3] D7, D24, D40, duration of storage of 7, 24 and 40, respectively.

[4] I: inoculation; T: storage temperature; L: duration of storage; I vs. T: I×T interaction; I vs. D: I×D interaction; T vs. D: T×D interaction.

Except with RM, figures followed by different superscripts in a row for inoculants, temperature and duration of storage differ significantly ($p<0.05$).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS: not significant.

and 0.31% DM for the same parameters. The pH was 5.62 and 3.32 for pre-silage material and silage, respectively, indicating a good fermentation process. The changes in pH, lactic acid and acetic acid contents of the silages as affected by the fungal inoculants under different storage temperatures and duration of storage are given in Table 1.

Effect of inoculants

There were no treatment effects on the DM of silages which were between 21.0 and 22.7%. The inoculants had no significant effect on the fermentation quality of PPS relative to the control silage, although numerically they reduced the pH and increased the lactic acid content. Acetic acid was equally not affected by inoculation.

Effect of storage temperature

The effect of storage temperature was obvious on the fermentation quality of PPS. Generally, the pH decreased ($p<0.01$) while lactic and acetic acid contents increased ($p<0.01$) with increase in storage temperature. There were no significant effects on lactic and acetic acid contents of the silages at 4 and 12°C. However, at 25°C both lactic and acetic acid contents increased ($p<0.01$).

Effect of duration of storage

The effect of duration of storage on the fermentation in PPS was similar to that of the storage temperature, decreasing ($p<0.01$) the pH and increasing ($p<0.01$) lactic acid production with progressive duration of storage. Acetic acid content was low at day 7, but increased and then decreased at 24 and 40 days of ensilage, respectively.

Starch and sugar contents in silages

The changes in starch and sugar contents are given in Table 1. Starch levels fluctuated with no clear tendency in effect of inoculation, storage temperature level and duration of storage. The only obvious effect, though not statistically significant, was that of PR and PA in numerical decreases relative to the control, an indication that the inoculants somewhat enhanced starch degradation in PPS. Effects of temperature and duration of storage were significant ($p<0.05$) for sugar but the inoculant effect was not. At 25°C the sugar level was low in comparison with that at lower temperatures of 4 and 12°C, while it increased significantly ($p<0.05$) at day 24 and 40, in comparison with day 7.

Interaction between inoculants, storage temperature and duration of storage

Interactions between inoculation and both temperature and duration of storage were absent for all measured parameters except for acetic acid (Table 1). The pH, lactic acid content and sugar concentration showed significant ($p<0.01$) interactions for both temperature and duration of storage.

Contrasts between effects of inoculation, storage temperatures and duration of storage

Contrasts between control and inoculated silages showed no statistical differences between R and A or the average response of inoculation relative to uninoculated silages (Table 2). There were, however, clear significant ($p<0.01$) effects between pre-silage material and silages with respect to temperature and duration of storage in all the measured parameters except for acetic acid and sugar concentrations. Of particular significance was the linear relationship ($p<0.001$, Table 2) between storage temperature/duration of storage for pH and lactic acid concentration. These relationships could be summarized in the following multi-linear equations:

$$pH = -0.046\,T - 0.021\,D + 5.10$$

$$R^2 = 0.81\ (p<0.01)$$

Table 2. Contrasts between control and inoculated silages and effects between pre-silage material and silages with respect to storage temperatures and duration of storage in potato pulp fermentation characteristics

	Contrast[1]											
	Inoculants			Temperature			Relationship		Duration of storage			Relationship	
	C:PR	C:PA	C:RA	RM:T4	RM:T12	RM:T25	Lin.	Quad.	RM:D7	RM:D24	RM:D40	Lin.	Quad.
Dry matter	NS	NS	NS	***	***	***	NS	NS	***	***	***	NS	NS
pH	NS	NS	NS	***	***	***	***	**	***	***	***	***	**
Lactic acid	NS	NS	NS	***	***	***	***	**	***	***	***	***	**
Acetic cid	NS	NS	NS	*	**	NS	NS	**	***	NS	NS	**	***
Starch	NS	NS	NS	*	*	***	*	NS	**	**	**	NS	NS
Sugar	NS	NS	NS	***	NS	*	*	***	***	***	NS	***	*

[1] C:PR: control silage vs. *Rhizopus oryzae* silage; C:PA: control silage vs. *Amylomyces rouxii* silage; C:RA: control silage vs. *R. oryzae* and *A. rouxii* silage.

RM:T4: RM:T12, RM:T25: pre-silage material vs. storage temperatures of 4, 12 and 25°C, respectively.

RM:D7: RM:D24, RM:D40: pre-silage material vs. storage duration of 7, 24 and 40 days, respectively.

Lin.: linear relationship; Quad.: quadratic relationship.

* p<0.05; ** p<0.01; *** p<0.001; NS: not significant.

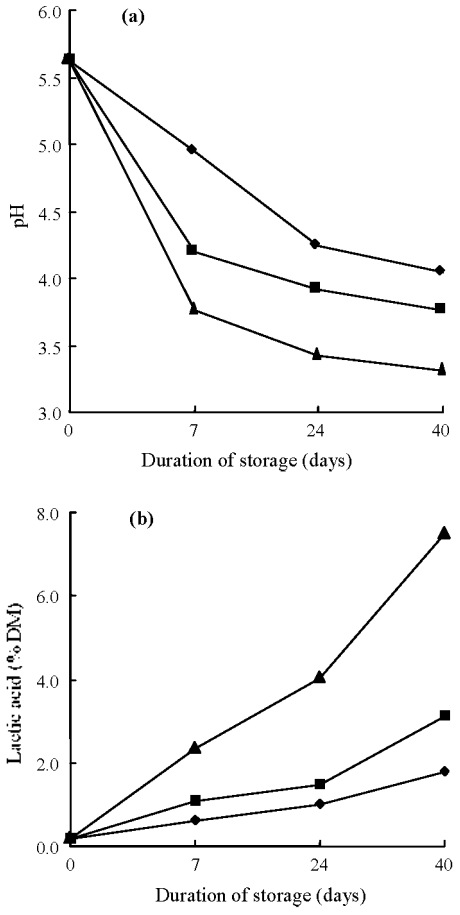

Figure 1. Change of pH (a) and lactic acid concentration (b) at various durations of storage under different storage temperatures in potato pulp silage. Values represent least square means (n = 81). (◆) 4°C; (■) 12°C; (▲) 25°C.

$$LA = 0.16\ T + 0.08\ D - 1.40$$
$$R^2 = 0.66\ (p<0.01)$$

where T, ensiling temperature (°C); D, duration of storage (days); LA, lactic acid content (% DM) and R, correlation co-efficient.

Starch content in pre-silage material significantly decreased ($p<0.05$) with storage temperature and duration of storage (Table 2). However, significant ($p<0.05$) but inconsistent relationships were observed for acetic acid and sugar concentrations.

DISCUSSION

This study was conducted to investigate the possible conditions necessary for improved fermentation in PPS inoculated with fungi as a follow up to previous research (Okine et al., 2005). The silo chosen for the study has been shown to be acceptable for ensiling without affecting the preservation quality (Ashbell et al., 2001). The chemical analysis of the pre-silage material and the final control silage showed that the changes in DM, pH, lactic and acetic acids were comparable to our previous study.

In the present investigation, the inoculant effect on PPS fermentation, especially lactic acid production, was minor, in confirmation of our previous results. We hypothesized that application of the fungi to potato pulp in conditions sufficient to maintain their growth would yield better inoculant response in fermentation end products as compared to the freeze-dried form employed in the previous study. This was built on the premise that the initial aerobic phase of the fungi was likely to influence their efficacy since oxygen is indispensable for fungal growth. Our results did not reveal a clear-cut and consistent effect of the fungi in lactic production in PPS. Potato pulp is characteristically dense and compaction or evacuation of the polyethylene bags prior to filling, though necessary for anaerobiosis, may have inhibited slow diffusion of oxygen into the surface and consequently fungal activity, thereby impairing the inoculants potential for lactic acid production. However, this assumption does not explain the high final

concentrations of lactic acid at the end of the period of investigation (Figure 1b). Mayer and Hillebrandt (1997) and later Saito et al. (2006), in investigations of microbial characteristics of raw potato pulp sampled from different varieties over a period of time, observed that the dominant microbe present was lactic acid bacteria (up to 10^8 CFU). This large load of lactic acid bacteria under favorable silo conditions such as sustained anaerobiosis and sufficient substrate availability would be enough to maintain fermentation even in the absence of a lactic acid fermentation stimulant. This report seems to support our previous conclusion (Okine et al., 2005) that potato pulp does ensile well without bacterial inoculants.

Ensiling is based on fermentation, which involves biological and enzymatic activity and as such is strongly influenced by temperature. There was a common trend in the fermentation pattern of PPS irrespective of the inoculants used. The pH reduced, while the lactic acid content increased, with rise in storage temperature and progressive duration of storage. The multi-linear equation elucidates this relationship. On the basis of the equation, to attain a pH of 3.50, for example, it takes only 21.4 days at a temperature of 25°C while it requires 49.9 and 67.0 days at 12 and 4°C, respectively. Similarly, to attain a lactic acid content of 3.5% DM it takes 11.3, 37.3 and 53.3 days at 25, 12 and 4°C, respectively. The pH was lower at elevated storage temperatures and extended periods of storage while lactic acid production accelerated at higher storage temperatures and extended periods of storage (Figure 1a, b). There was a higher correlation for pH decrease than for the rise in lactic acid production as evident from the correlation co-efficients (R^2 = 0.81 vs. 0.66). The insignificant differences in lactic acid content between 4 and 12°C on one hand and also at days 7 and 24 (Table 1) do indicate the relevance of a combined effect of both storage temperature and duration of storage on PPS fermentation.

Although the acetic acid content fluctuated inconsistently, the increase ($p<0.01$) at 25°C followed an increase in lactic acid content (Table 1), reflecting the conversion of the latter to the former and/or the permeability of some oxygen into the bags, in agreement with Ashbell et al. (2001). These effects, however, did not adversely affect the quality of the silages and may have protected them from development of butyric acid and molds, which were not detected in any of the silages. The starch content was not affected by the inoculation, temperature or duration of storage. The hypothesis that R and A enhance potato pulp starch degradation or conversion to sugar for use as substrate for lactic acid fermentation could not be validated by the present results. Although inoculated silages had numerically (Table 1) higher values relative to the control, starch in potato pulp was degraded with/without the inoculants. The slow degradation of potato starch (Monteils et al., 2002) may be responsible for this, or during fermentation enough sugar was being produced from hydrolysis of other carbohydrate sources such as pectin (De Man, 1957; Okine et al., 2005) through the enzyme pectinase (produced by the fungi) in amounts sufficient to maintain fermentation, and this obviated the need to hydrolyze starch. The significant decrease in sugar levels (from 12 to 25°C) indicated that high ensiling temperatures increased the rate of sugar consumption as substrate for lactic acid production.

CONCLUSION

These data suggest that the fermentation quality of potato pulp silage is influenced most by ensiling temperatures and the duration of storage and least by inoculation with fungal inoculants. The use of the *Rhizopus oryzae* and *Amylomyces rouxii* in potato pulp silage fermentation should be weighed against their cost and other nutritional benefits.

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