Chemical composition and protein enrichment of orange peels and sugar beet pulp after fermentation by two *Trichoderma* species

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Summary

The present experiment aimed at increasing orange peel and sugar beet pulp protein content through solid-state fermentation by *Trichoderma reesei* and *Trichoderma viride*. *In vitro* digestibility and changes in the chemical composition of the fermented products were determined after seven days of fungal cultivation using gas production tests. The cultivation of *T. reesei* and *T. viride* on orange peels decreased neutral detergent soluble content (P<0.01) and increased cellulose, hemicellulose and lignin contents (P<0.01). Changes in fiber fractions were found to be more pronounced with *T. viride*. The cultivation of *T. reesei* and *T. viride* on sugar beet pulp increased neutral detergent soluble content (P<0.01) and decreased cellulose and hemicellulose contents (P<0.01). These changes were more pronounced with *T. reesei*. The cultivation of *T. reesei* or *T. viride* on orange peels or sugar beet pulp increased crude protein content (P<0.01) compared with the unfermented materials; however, the increase was more pronounced for orange peels fermented with *T. viride* when corrected for weight loss (P<0.05). After 24 and 48 h of incubation, significant decreases in cumulative gas production (P<0.01) were observed in fermented sugar beet pulp and orange peels compared with the unfermented materials. Fungal treatment of orange peels and sugar beet pulp reduced the digestibility of *in vitro* organic matter, metabolizable energy and average fermentation and gas production rates (P<0.01). The data showed that seven days of solid-state fermentation of orange peels and sugar beet pulp by *T. reesei* or *T. viride* can increase their crude protein content.

Key words: Fermentation, *Trichoderma viride*, *Trichoderma reesei*, Orange peel, Sugar beet pulp

Introduction

With the increasing world population, the demand for feed and protein sources for livestock has also increased (Coward-Kelly *et al*., 2006). Utilization of by-products of food processing industries in the diet of livestock has several advantages because they are inexpensive, fairly abundant in most places, and if properly managed, can alleviate the problems of waste disposal and management (Bampidis and Robinson, 2006). Most of these by-products are rich in carbohydrates but very low in proteins, therefore using them efficiently as animal feed depends on further processing to increase their nutritional value.

Solid-state fermentation (SSF), defined as the growth of microorganisms on wet solid substrates in the absence of free water (Pérez-Guerra *et al*., 2003), has the potential to increase the protein content of agricultural by-products. It is a low energy-demanding technology, produces less wastewater, reduces the risk of contamination due to low water availability and is environmentally safe (Manpreet *et al*., 2005).

Fungal treatment for increasing protein content in by-product feed has been widely attempted (Mahesh and Mohini, 2013). Members of the *Trichoderma* genera, a group of filamentous fungi, are well suited to be used in SSF as they easily spread over the solid matrix and penetrate into the solid substrate (Manpreet *et al*., 2005). In addition, they successfully colonize their habitat and dominate their competitors (Schuster and Schmoll, 2010). The effective protein production capability of *T. viride* on cassava peel (Ogbonnaya *et al*., 2013), peanut hulls (Abo-Donia *et al*., 2014) and pineapple waste (Omwango *et al*., 2013), and *T. reesei* on corn stalks (Omer *et al*., 2012) and banana peels (Baral and Adhikari, 1998) has been demonstrated previously.

Orange peels (OP), which are the main by-product of the citrus processing industry, are rich in pectin, cellulose, and hemicellulose, but poor in protein (5.8%), and constitute approximately 50% of the fresh fruit weight (Bampidis and Robinson, 2005; Mamma *et al*., 2008). This by-product has a high potential degradable dry matter in the rumen with a degradation rate of approximately 3.1% h^{-1}, suggesting its high energetic value for ruminants (Silva *et al*., 1997).

Sugar beet pulp (SBP), the primary by-product of the sugar refining industry, is the residue remaining after sucrose extraction from sugar beets, containing 22–30%

cellulose and 22–30% hemicellulose (Zheng *et al*., 2011), 25% pectin, fat (1.4%), protein (10.3%), ash (3.7%) and lignin (5.9%) (Sun and Hughes, 1999). Approximately 250,000 tons of SBP is produced annually in Iran, mainly in Fars, Mazandaran, Khuzestan, Kerman, Isfahan, Khorasan and Zanjan provinces (Najafi *et al*., 2009). Because of its high water (60–90%) and monosaccharide contents, SBP is usually dried to reduce the risk of spoilage and microbial contamination (Zheng *et al*., 2011); however, drying is a costly process and not an ideal method for SBP preservation.

SSF can be used as an effective and inexpensive method for preserving the high moisture content of by-products such as OP and SBP, and at the same time increasing their nutritional value. The objective of this study was to evaluate the possibility of increasing the protein content of OP and SBP by fungal cultivation through SSF, and to determine the compositional changes and *in vitro* gas production kinetics of the fermented products.

Materials and Methods

Substrate preparation

Dried SBP was obtained from Marvdasht Sugar Factory, Shiraz, Iran. Fresh OP batches (exclusive of seeds), provided by three local citrus processors in Shiraz, were sun-dried for 7 days. Dried SBP and OP were hammer-milled to pass through a 1-mm screen. Ground-bulked SBP or OP was then sub-divided into nine portions. Experimental treatments consisted of unprocessed SBP and OP, and SBP and OP processed with *T. reesei* or *T. viride* strains. Each treatment was replicated three times.

Strain and spore preparation

Trichoderma reesei (PTCC 5142) and *Trichoderma viride* (PTCC 5157) strains were obtained from Persian type culture collection (PTCC), Tehran, Iran. For stock culture preparation, a freeze-dried culture of *T. reesei* was reactivated and maintained on Potato Dextrose Agar (PDA) in Petri plates at 24°C. The *T. viride* strain, provided as live culture, was maintained at 26°C on sterilized wheat sprouts. After good sporulation (10^6 spores/ml), spores were harvested aseptically by washing the Petri plate with sterile water (10 ml). The resultant spore suspension was then used as the inoculum. Spore concentration was determined using a hemocytometer under a phase-contrast microscope.

Mineral salt medium

The mineral salt medium contained 2.0 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 1 g/L peptone, 0.6 g/L $MgSO_4.7H_2O$, 0.4 g/L $CaCl_2.2H_2O$, 0.3 g/L urea, 2.7 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4.H_2O$, 1.4 mg/L $ZnSO_4.H_2O$ and 3.7 mg/L $CoCl_2.6H_2O$ (Mandels and Weber, 1969). The medium pH was adjusted at 4.8 using 0.1 N HCl, and autoclaved at 121°C for 15 min.

Solid-state fermentation (SSF)

The fermentation process was performed inside a 500-ml Erlenmeyer flask containing 40 g substrate moistened with 15 ml of a mineral salt medium. The flasks containing moistened pulps were autoclaved at 121°C for 15 min. To ensure that pulps were free of contamination, wet pulps were autoclaved in a two-step process. In the first step, they were autoclaved and allowed to cool down to room temperature, then autoclaved again after 24-h. This helped the spores that might have survived the first sterilization process to germinate (Tuyen *et al*., 2012), thus eliminating the risk of unwanted microorganisms. After autoclaving, the flasks were maintained at room temperature for 7 days. No contamination was detected after the two-step autoclaving of SBP and the one-step autoclaving of OP. The autoclaved flasks were cooled to room temperature and the substrate was inoculated aseptically with a spore suspension (10 ml). The moisture content for SSF was adjusted at 70 g/kg. The flasks were cotton bunged to allow air exchange and incubated as static cultures for 7 days in a laboratory incubator (Fisher Isotemp Model 349 Incubator) set at 24°C for *T. reesei* and 26°C for *T. viride*. Autoclaved un-inoculated flasks were used as the control. At the end of fermentation period, the content of each flask was oven-dried (55°C for 48 h), homogenized and stored in a zip-locked plastic bag at 4°C until further analysis. Weight loss was calculated by subtracting the weight of the fermented substrate from the weight of the dried un-inoculated (control) substrate.

Chemical analysis

Crude protein (CP, N × 6.25, method ID 984.13), dry matter (DM, method ID 930.15), and ash (method ID 942.05) were determined according to AOAC (1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF), inclusive of residual ash, were determined by ANKOM A200 Filter Bag technique (ANKOM model 200; F-57, ANKOM Technology Corp., Macedon, NY) according to Van Soest *et al*. (1991). Acid detergent lignin (ADL) content was measured by cellulose solubilization with 72% sulfuric acid in an ANKOM Daisy^{II} Incubator (ANKOM Technology, Fairport, NY, USA) according to Ankom protocol. Concentrations of neutral detergent solubles (NDS), hemicellulose, and cellulose were calculated by difference as "1000–NDF," "NDF–ADF" and "ADF–ADL", respectively.

In vitro gas production measurement

Gas production (GP) parameters of unfermented and fungal-fermented materials were determined using an *in vitro* GP test. Rumen fluid was taken before morning feeding from two fistulated non-lactating Holstein cows (660.0 ± 11.2 kg body weight) fed a diet of 30% wheat straw, 40% concentrate mix, and 30% alfalfa hay supplemented with mineral/vitamin premix. Procedures for preparing rumen fluid and artificial saliva have been described previously by Ahmadi *et al*. (2013). Sixty ml of rumen fluid/buffer mixture was dispensed into a vial

containing approximately 400 mg feed sample, flushed with CO_2 for 10 sec, immediately sealed with a butyl rubber stopper and incubated inside a shaking water bath at 39°C. Gas volume was measured using a water displacement apparatus (Fedorak and Hrudey, 1983). Four vials containing only rumen fluid-buffer mixture were used as blanks and the net GP (ml/g DM) was calculated. At 48 h of *in vitro* incubation, the pH of the digesta was measured (Jenway Model 3510, Camlab, Cambridge, UK).

Cumulative GP data were fitted with a NLIN procedure (SAS, 2003) to the following non-linear model (Ørskov and McDonald, 1979):

(1) $P = b\ (1 - \exp^{-c(t-L)})$,

where
P: GP (ml) at time t
b: Asymptotic GP (ml/g DM)
c: GP rate constant (1/h)
t: Incubation time (h)
L: Lag time which was either zero or very negligible and not reported

Metabolizable energy (ME) and organic matter digestibility (OMD) were estimated using the following equations (Menke and Steingass, 1988):

(2) ME (MJ/kg DM) = $0.136 \times GP_{24} + 0.057 \times CP + 2.20$

and

(3) OMD (g/100 g DM) = $14.88 + 0.889 \times GP_{24} + 0.45 \times CP + 0.0651 \times XA$,

where
GP_{24}: 24-h net GP (ml/200 mg DM)
CP: Crude protein (% DM)
XA: Ash content (% DM)

Average fermentation rate (AFR, ml/g DM/h) was calculated according to the following equation (Hervás *et al*., 2005):

(4) $AFR = \dfrac{(A \times c)}{(2 \times \ln 2) + (c \times L)}$

where
A: The asymptotic GP (ml gas/g DM) equivalent to b obtained from equation (1)
L: Lag time (h)
c: GP rate constant (1/h)

Aflatoxin detection

Total aflatoxin was measured using high-pressure Liquid Chromatography (Agilent 1100 model, USA) equipped with an auto-sampler G1367A,fluorescence detection at 435 nm and excitation of 365 nm. After proper dilution and filtration, the sample extract was passed through a column packed with octadecylsilanized silica gel (ODS) column (YMC-Pack Pro C18, 4.6 mm inside diameter, 25 cm length, 5 µm particle diameter; YMC Co., Ltd., Kyoto, Japan). The methanol/acetonitrile/water (4:3:3 v/v/v) was used as a mobile phase. Flow rate was set at 1 ml/min.

Statistical analysis

Data obtained from each feed ingredient were analyzed using one-way analysis of variance with SPSS software (Ver. 16.0.0, 2007, SPSS Inc., Chicago, IL, USA). The level of significance was set at $P<0.05$. Mean comparison was performed by Duncan's multiple range test. The statistical model for the data analysis was:

$Y_{ij} = \mu + T_i + e_{ij}$

where
Y_{ij}: Observation j in treatment i
μ: The general mean
T_i: Effect of i^{th} treatment (i = 1 to 3)
e_{ij}: The residual error (j = 1 to 3)

Results

No aflatoxin contamination was detected in either unfermented or fungal-fermented substrates.

Chemical composition and weight loss of fungal-fermented substrates

The effects of fungal SSF on chemical composition and weight loss in OP and SBP are shown in Table 1. Fungal cultivation significantly increased the CP content in OP or SBP ($P<0.01$). Increase in the CP content was greater with *T. viride* compared with *T. reesei* ($P<0.01$). The increase in CP corrected for weight loss in OP fermented by *T. viride* was greater than *T. reesei* ($P<0.05$). Weight loss was not different for OP or SBP with either fungal species ($P>0.05$). The increase in CP corrected for weight loss in SBP was not different between samples fermented by *T. reesei* or *T. viride* ($P>0.05$).

Cultivation of *T. reesei* and *T. viride* on OP decreased NDS ($P<0.01$) and increased cellulose, hemicellulose and ADL contents ($P<0.01$). Changes in fiber fractions were more pronounced with *T. viride*. Cultivation of *T. reesei* and *T. viride* on SBP increased NDS ($P<0.01$) and decreased cellulose and hemicellulose contents ($P<0.01$). These changes were more pronounced with *T. reesei*. In fungal-fermented materials, greater increases in ADL concentrations were observed in feed samples fermented by *T. viride*.

Gas production kinetics

The GP parameters for unfermented and fermented OP and SBP are shown in Table 2. Reductions in the GP rate constant (*c*) ($P<0.01$), GP at 24 and 48 h post-incubation, OMD and ME were observed because of the fungal treatment of the materials. Reductions in GP parameters were greater with *T. viride* compared with *T. reesei* ($P<0.01$). Lag time was not affected by fungal treatment ($P>0.05$). The increase in pH after 48 h of incubation due to fungal treatment was significant for both materials ($P<0.05$). Gas production in fermented OP and SBP was lower than that of untreated materials. Greater reduction in gas volume after 24 or 48 h of incubation was recorded in the substrates fermented by *T. viride*.

Table 1: Effect of solid-state fermentation of OP and SBP by *T. viride* and *T. reesei* on chemical composition and weight loss

Item	Orange peel			SEM[1]	P-value	Sugar beet pulp			SEM[1]	P-value
	Unfermented	*T. viride*	*T. reesei*			Unfermented	*T. viride*	*T. reesei*		
g/kg DM unless stated										
Weight loss	-	139	127	5.7	0.33	-	123	106	5.1	0.09
Crude protein	57.2^{c}	129^{a}	111^{b}	10.8	<0.01	91.8^{c}	175^{a}	166^{b}	2.7	<0.01
Increase (%)	-	126^{a}	94.1^{b}	7.74	<0.05	-	90.6	80.8	2.94	0.10
Corrected increase (%)[2]	-	108.1^{a}	82.1^{b}	5.13	<0.05	-	79.5	72.3	2.83	0.29
Neutral detergent soluble	795^{a}	626^{c}	704^{b}	24.4	<0.01	548^{c}	653^{b}	696^{a}	22.2	<0.01
Decrease or increase (%)	-	-21.4^{a}	-11.4^{b}	2.14	<0.01	-	19.2	27.0	1.08	0.05
Corrected decrease or increase (%)[2]	-	-18.4^{a}	-9.99^{b}	2.29	<0.01	-	16.8	24.1	0.64	0.37
Hemicellulose	71.2^{c}	150^{a}	135^{b}	12.2	<0.01	223^{a}	147^{b}	129^{c}	14.6	<0.01
Increase or decrease (%)	-	111	89.6	6.42	0.10	-	-34.1^{b}	-42.2^{a}	2.11	0.01
Corrected increase or decrease (%)[2]	-	95.3	78.2	6.02	0.20	-	-29.9^{b}	-37.7^{a}	1.49	0.02
Cellulose	128^{c}	212^{a}	153^{b}	12.4	<0.01	193^{a}	157^{b}	138^{c}	8.2	<0.01
Increase or decrease (%)	-	65.6^{b}	19.5^{a}	10.30	<0.01	-	-18.7^{b}	-28.5^{a}	2.43	0.01
Corrected increase or decrease (%)[2]	-	56.5^{b}	17.1^{a}	8.98	<0.01	-	-16.4^{b}	-25.5^{a}	1.42	<0.05
Acid detergent lignin	5.80^{c}	12.0^{a}	8.00^{b}	0.84	<0.01	36.0	43.0	37.0	2.32	0.49

[a-c] Means within each row with different superscripts for each feed ingredient are significantly different (P<0.05). [1] Standard error of means (n = 9), and [2] Corrected for weight loss

Table 2: Effect of solid-state fermentation of OP and SBP by *T. viride* and *T. reesei* on *in vitro* gas production parameters

Parameters	Orange peel			SEM[1]	P-value	Sugar beet pulp			SEM[1]	P-value
	Unfermented	*T. viride*	*T. reesei*			Unfermented	*T. viride*	*T. reesei*		
OMD (g/kg DM)	746^{a}	577^{c}	665^{b}	17.7	<0.01	778^{a}	478^{c}	596^{b}	34.2	<0.01
ME (MJ/kg DM)	11.2^{a}	8.57^{c}	9.93^{b}	0.29	<0.01	11.7^{a}	6.98^{c}	8.80^{b}	0.47	<0.01
pH[2]	6.19^{a}	6.68^{b}	6.62^{b}	0.05	<0.01	6.41^{a}	6.73^{b}	6.68^{b}	0.04	<0.01
GP (ml/g DM)										
at 24 h	320^{a}	207^{c}	261^{b}	12.0	<0.01	329^{a}	139^{c}	208^{b}	19.1	<0.01
at 48 h	345^{a}	244^{c}	291^{b}	10.8	<0.01	391^{a}	222^{c}	278^{b}	17.2	<0.01
Fermentation kinetics										
b (ml/g DM)	344^{a}	261^{b}	288^{b}	11.1	<0.01	385^{a}	363^{b}	287^{c}	11.4	<0.01
AFR (ml/g DM/h)	28.2^{a}	10.8^{c}	21.4^{b}	1.82	<0.01	23.9^{a}	4.51^{c}	11.9^{b}	1.94	<0.01
c (1/h)	0.114^{a}	0.057^{c}	0.103^{b}	0.006	<0.01	0.087^{a}	0.017^{c}	0.058^{b}	0.007	<0.01

[a-c] Means within each row with different superscripts for each feed ingredient are significantly different (P<0.05). [1] Standard error of means (n = 9), [2] At 48 h incubation

Discussion

Increases in CP contents of the fermented materials in the current experiment are consistent with Iconomou *et al*. (1998) who reported the CP content of SBP fermented with *T. reesei* to have reached 226 and 248 g/kg after 4 and 5 days of cultivation at 800 g/kg moisture content, respectively. In another study on the protein enrichment of banana peels through SSF by *T. reesei*, CP was raised substantially from 58 to 135 g/kg after 72 h (Baral and Adhikari, 1998). The CP content of cashewnut bran after 15 days of SSF by *T. viride* increased from 40 to 78 g/kg at the expense of cellulose reduction from 325 to 152 g/kg (Eyini *et al*., 2002). The cultivation of *T. viride* on a mixture of palm kernel cake and rice bran increased crude protein content by 29.6% (Yana *et al*., 2010). In an *in vivo* study, lambs fed with *T. viride*-fermented rice straw and corn stalk had a higher nitrogen balance. Cassava peel treated with *T. viride* contained all essential amino acids; but the chemical score of the essential amino acids revealed that methionine was the limiting amino acid (Ezekiel *et al*., 2010).

After a 14-d SSF with *T. viride*, the protein content of brewer's dried grains, rice bran, palm kernel meal, and corn bran increased by 87, 68, 32 and 61%, respectively, whereas fiber content decreased by 35, 40, 37 and 38%, respectively (Iyayi and Aderolu, 2004). The increase in protein content of fungal-fermented materials is caused by nitrogen capturing by aerobic fermentation and urea from the mineral salt medium (Salman *et al*., 2008). *Trichoderma* spp. have cellulase and hemicellulase but no ligninase activities (Gritzali and Brown, 1979). This leads to the utilization of simple sugars, cellulose and hemicelluloses in the pulp, thereby increasing ADL concentrations.

Weight loss in SBP and OP after fungal fermentation (on average 115 and 133 g/kg DM, respectively) can be due to fungal consumptions of carbon sources and the production of carbon dioxide during fermentation. Smits *et al*. (1996) investigated carbon balance in wheat bran fermented with *T. reesei* and observed a weight loss of 220 g/kg after 125 h of fermentation. This weight loss was attributed to both moisture loss and substrate consumption. Weight loss due to carbon consumption was 100 g/kg for wheat bran. In another study, weight losses of 187 and 243 g/kg were reported for palm kernel cake fermented by *T. longibrachiatum* and *T. harzianum*, respectively (Iluyemi *et al*., 2006).

Several factors may have contributed to lower amounts of gas released from the fermented substrates. A recent study investigated the *in vitro* digestibility of fungal-fermented palm kernel cake (Ramin *et al*., 2010). The lower amount of gas released from palm kernel cake during 10-d fermentation by *Aspergillus niger* and *Rhizopus oryzae* compared with unfermented samples

was hypothesized to be due to the production of statins by fungus; hydroxymethylglutaryl-SCoA was reported to inhibit methanogens, but had no effect on cellulolytic bacteria (Wolin and Miller, 2006). Fungal preference for consuming simple sugars in OP and the subsequent increase in fiber concentration may be a factor causing decreased GP (Vaccarino *et al*., 1989). Different patterns of GP from proteins and carbohydrates can be another reason for the reduced GP. As reported by Cone and Van Gelder (1999), the amount of gas produced from casein fermentation was 32% of that produced from potato starch; with every 10 g/kg increase in protein content, a reduction of 2.48 ml gas/g DM was observed. Therefore, a lower amount of gas released from fermented pulps may be due to the fungal conversion of carbohydrate-rich pulp into protein-rich material.

The results of the present study revealed a substantial increase in CP content despite the weight loss in solid-state fermented OP and SBP using both *T. reesei* and *T. viride*. The fungi behaved differently when used on OP or SBP as substrates. When cultivating *T. reesei* and *T. viride* on OP, NDS were utilized and fiber fractions were accumulated, whereas with SBP, fiber fractions were used and NDS were accumulated. Nevertheless a reduction in *in vitro* gas production was also observed, which can be due to the production of fungal statins in addition to the conversion of fibrous fractions into proteins.

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