Protein-enriched cassava root pulp as partial replacement for fish meal in diets for growing pigs

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Abstract

Two experiments were carried out to study: (i) the effect of urea levels on conversion of NPN to true protein in a solid-state fermentation of cassava pulp: rice bran (70:30 DM basis) with yeast and diammonium phosphate (DAP); and (ii) how this would affect the performance of growing-fattening pigs fed the yeast enriched pulp (PECP) as partial replacement of fish meal in the diet. In experiment 1, the two factors in a 2*2 factorial design with 3 replications were: urea concentration: 0, 0.5, 1.0, 1.5 and 2% (DM basis). (DAP was constant at 1% of substrate DM); and fermentation time (0, 3, 5 and 7 days). In experiment 2 the treatments in a 4-month growing-fattening trial with 25 pigs were: 0, 25, 50, 75 and 100% replacement of fish meal protein by protein from PECP (equivalent to PECP levels of 0, 3.9, 7.7, 11.7 and 15.6% of diet DM from 15 to 50 kg live weight; and 0, 4.5, 8.9, 13.4 and 17.8% of DM from 50 to 100 kg live weight.

Experiment 1: After 7 days of fermentation, the level of true protein reached a range of 12.3 to 12.5% in DM when urea levels were in the range of 0.5 to 2% of the substrate DM. There were no differences between urea levels of 0.5 and 2%. Comparable values for crude protein were in the range 24 to 27.5% in DM. These increases in concentrations of “true” and “crude” protein were associated with a loss of some 30% of the substrate DM indicating that approximately 3 kg of substrate DM were fermented to produce 1 kg of true (yeast) protein. The overall conversion rate of crude protein (from urea and DAP) to true protein was of the order of 60% when the urea level was between 0.5 and 2% of the substrate DM. The best conversion rate of crude to true protein was with 0.5% urea in substrate DM.

Experiment 2: With 25% fish meal protein replacement (4% PECP in the diet DM), feed intake was reduced, live weight gain did not differ, and DM feed conversion tended to be improved. At higher proportions of PECP in the diet, there was a linear depression in live weight gain (overall reduction of 25%) as protein from PECR replaced that from fish meal in the range of 25 to 100% of the fish meal protein (equal to average PECP levels of from 4 to 16.7% in diet DM). There was no consistent trend in the effect of PECP level on feed conversion. It is suggested that the reduction in live weight gain with more than 4% PECP in the diet DM may be related to sub-acute toxicity caused by residual NPN compounds in the PECP because of incomplete conversion of the NPN to yeast protein.

Key words: *fermentation, non-protein-nitrogen, true protein, Pichia kudriavzevii*

Introduction

In Vietnam, cassava is an important food-crop and is commonly planted in upland areas of the country. In Thua Thien Hue province, cassava is cultivated on sloping land due to its tolerance to poor soil conditions. In the past, cassava was often used as feed for livestock, but at present it is used mainly for producing starch. After harvesting, most of the cassava is sold to the cassava starch factory in Phong An commune, Phong Dien district, Thua Thien Hue province. Only one third of the cassava root biomass is extracted as starch leaving two thirds as cassava by-product known as cassava pulp. In the harvesting season, 100-150 tonnes of cassava pulp are discharged per day, which causes environmental problems as it decays rapidly.

At present, the cassava pulp is utilized to a limited extent by farmers, but its use is constrained by the presence of cyanogenic glucosides which are converted to toxic HCN when consumed by animals. The low protein content is another limiting factor.

However, it is possible to reduce the content of HCN precursors and to improve the protein content in carbohydrate-rich industrial byproducts by solid-state microbial fermentation (Brook et al 1969; Obahdina et al 2006; Aro et 2008; Thongkratok et al 2010).

The application of this technology to cassava pulp has been studied recently in Lao PDR by Manavanh et al 2016; Vanhnasin and Preston 2016; Vanhnasin et al et al (2016) and Sengxayalth and Preston (2017a,b). These researchers reported an increase in true protein from 2 to 7-12% in dry matter (DM) of the cassava pulp (cassava root in the case of Vanhnasin et al 2016) and that the protein-enriched product (PECP) could provide up to 28% of the dietary protein in a diet based on cassava pulp (or ensiled root), replacing ensiled taro foliage (Vanhnsin and Preston 2016) or soybean meal (Sengxayalth and Preston 2017b). However, higher proportions of the protein-enriched feed in the diet led to reduced growth performance with almost no growth at 100% replacement of the taro/soybean protein. It was reported that only 50-60% of the added NPN (from urea and DAP) was recovered as true protein which implied that more than one third of the original NPN remained, possibly in the form of ammonium salts or intermediary products formed in the process of yeast growth. It was proposed (Sengxayalth and Preston 2017b) that the improvement in growth rate with 30% PECP protein in the diet was the result of amino acid synthesis by bacteria in the small intestine using the residual dietary ammonia as substrate (Colombus et al 2014); and that at higher levels of substitution of PECP the severe depression in feed intake and in growth was due to the toxicity caused by the residual NPN compounds in the fermented pulp/root.

Two experiments were carried out to determine if the urea levels in the solid-state fermentation of cassava pulp with yeast could be reduced, and how this would affect: (i) the conversion of NPN to true protein; and (ii) the performance of growing-fattening pigs fed the yeast enriched pulp.

Experiment 1: Protein-enrichment of cassava root pulp by solid state fermentation with yeast, urea and diammonium phosphate (DAP)

Materials and methods

Materials

Cassava pulp was collected from the cassava starch processing factory in Phong Dien district, Thua

Thien Hue province. Rice bran was purchased from a feed shop in Hue city.

Microorganisms and inoculum preparation

The yeast *Pichia kudriavzevii* was obtained from the laboratory of Animal Science and Veterinary Medicine, Hue University. It was cultured on YPD (Yeast Extract Peptone Dextrose) medium at 37 ^{o}C in bottles placed on a reciprocal shaker operated at 150 rpm for 3 days, after which it was stored at 4^{o}C. The *Pichia kudriavzevii* was harvested by centrifugation at 3025 x g for 30 minutes at 6^{o}C and the pellet resuspended in acetate buffer (0.1 M, pH 5.7) containing 30% glycerol (w/w). This suspension was stored at -70^{o}C. Then 1.0g of the yeast was transferred to a solution containing 20% molasses (w/v), 2% urea (w/v) and 1% diammonium phosphate (DAP) (w/v). The suspension was then incubated at room temperature, with shaking at 150 rpm during 3 days. This suspension, containing approximately 10^6 CFU/ml of *Pichia kudriavzevii*, was used to ferment the cassava pulp.

Fermentation procedure

The two factors in a 2*2 factorial design with 3 replications were: urea concentration: 0, 0.5, 1.0, 1.5 and 2% (DM basis); and fermentation time (0, 3, 5 and 7 days).

The substrate was cassava pulp meal and rice bran mixed in a 70:30 (w/w) ratio. The different levels of urea and the DAP were dissolved in 97 liters of water and together with 3 liters of the suspension of *Pichia kudriavzevii* were mixed with the substrate. The mixture (Table 1) was put in trays at a depth of approximately 5 cm and covered by plastic wrap for fermentation at room temperature during 7 days (Photo 1).

Photo 1. Fermenting the substrate

Table 1. Composition of the substrates

Urea, kg	DAP, kg	Cassava pulp, kg	Rice bran, kg	PK suspension, liters	Water, liters
0	1	70	30	3	97
0.25	1	70	30	3	97
0.5	1	70	30	3	97
0.75	1	70	30	3	97

1.0	1	70	30	3	97

Chemical analysis

DM, crude and true protein, ash were analyzed in unfermented and fermented cassava pulp according to AOAC (1997). True protein was determined after treatment of the samples with trichloroacetic acid (TCA) to precipitate the protein (AOAC 1997).

Statistical analysis

The data were analyzed by the GLM option in the ANOVA program of the Minitab (2016) software. Sources of variation were levels of urea, times of fermentation, the interaction levels*times and error.

Results and discussion

Effect of fermentation on the content of crude protein

Figure 1 is based on the analytical data (Table 2) for 0, 3, 5 and 7 days of fermentation (FD 0 to 7). The column ‘Calc CP’ has the values calculated for the theoretical crude protein in the subsrates derived from the addition of urea (N*6.25 = 288% CP in DM) and DAP (N*6.25=128% CP in DM) assuming the mixture of cassava pulp and rice bran contained 6% CP in DM (Table 1). (thus CP in DM of substrate with 2% urea and 1% DAP= 6.0+2*2.88+1*1.28= 13.0 CP in DM)

Table 2. Mean values for true (TP) and crude protein (CP) in the substrates after 0, 3, 5 and 7 days of yeast fermentation (all values on DM basis) with urea levels of 0 to 2% (1% DAP in all treatments)

Urea %	0 days		3 days		5 days		7 days	
	TP	CP	TP	CP	TP	CP	TP	CP
0	3.53	6.60	4.05	7.15	5.56	8.34	6.36	8.76
0.5	5.46	13.1	7.70	17.0	11.3	21.9	11.6	24.2
1	5.63	12.5	7.53	22.9	10.1	24.5	12.2	26.6
1.5	4.73	14.1	8.24	20.5	11.5	26.3	12.7	27.0

2	5.14	15.2	7.68	19.5	11.2	26.2	12.3	27.0

There were major differences between the theoretical levels of crude protein based on the levels of urea and DAP and those determined by chemical analysis. For all fermentation times (including time zero but after adding the urea and DAP), the values determined by analysis were always higher than the theoretical levels; and the extent of the difference increased with the length of the fermentation (Figure 1). From 3 to 7 days the the levels of CP were twice as high as on day zero. It was especially notable that these differences were only observed when urea was included in the substrate.

Figure 1. Changes in crude protein in the substrate according to level of added urea and duration of ferrmentation

The analysis of chamges in DM content of the substrate with time of fermentation (Figure 2) shows that when urea was present, there was a reduction in DM content from 30 to 22%, and that this was independent of the level of urea in the range of 0.5 to 2% urea in substrate DM. In a similar solid-state fermentation of cassava root, with yeast, urea and DAP, Manivanh et al (2016) showed that these changes in DM content were the result of a loss of 30% of the substrate (determined by weighing the substrate and analysing for DM% before and sfter fermentation).

A 30% loss of sustrate would have the effect of “concentrating” the yeast protein as a percent of the substrate DM by some 30-40%. However, at the highest urea level of 2% in substrate DM, the CP increased to 27% in DM - - an increase of 100%. Therefore not all the increase in CP is accounted for by the loss of substrate in the conversion of carbohydrate to yeast and carbon dioxide. There is recent evidence that some yeasts can fix atmospheric nitrogen (Nwe Ni Win Htet et al 2013); although earlier reports considered this to be unlikely (Millbank 1969). However, in the absence of urea there was no increase in crude protein with fermentation time. There is no apparent explanation for this difference in N-enrichment of the substrate in the absence and presence of urea.

Figure 2. Change in DM content after 7 days fermentation acccording to level of urea added to the substrate

Effect of fermentation on the content of true protein

After 7 days of fermentation, the proportion of the “crude” protein converted to “true” protein was of the order of 30-40% (Figure 3) when the urea level was between 0.5 and 2.0% in substrate DM. The greatest increase was with the addition of 0.5% urea as compared with zero urea (but with 1% DAP which was present at all urea levels). These rates of conversion of “crude” to “true” protein were lower than the values of 60-65% reported by Vanhnasin et al (2016), who fermented fresh cassava root with 3% urea, 1% DAP and 2% of yeast (*Saccharomyces cerevisiae*), and Sengxayalth and Preston (2017a) who fermented cassava pulp with similar levels of yeast, urea and DAP. However, it is probably more appropriate to compare actual levels of true protein which reached a maximum of 12.5% in DM in the present experiment, similar to the level of 12.5% in DM reported by Sengxayalth and Preston (2017a) and higher than the values of 7.3 and 7.8% in DM reported by Vanhnasin et al (2016) and Manivanh et al (2016). In the two experiments where levels of 12.5% true protein in DM were achieved, the substrate was either 100% cassava pulp (Sengxayalth and Preston 2017a) or 70% cassava pulp and 30% rice bran as in the present experiment. Where lower levels of true protein were reached the substrate was fresh cassava root (Vanhnasin et al 2016; Manivanh et al 2016).

Figure 3. Changes in true and crude protein in the substrate according to level of added urea and duration of ferrmentation

There appeared to be no advantage in raising the urea level above 0.5% of the substrate DM, nor in extending the fermentation beyond 5 days, in terms of the production of true protein (Figure 4). On the contrary, the higher levels of urea would appear to have negative consequences for feeding animals as the residual NPN potentially could give rise to ammonia toxicity as was hypothesized by Sengxayalth and Preston (2017b).

Figure 4. Changes in true protein in the substrate according to level of added urea (% in DM) and duration of ferrmentation

The overall result of the fermentation indicates that, on the positive side, 1 kg of yeast protein is produced from approximately 3 kg of fermentable substrate in the form of a 70:30 mixture of cassava pulp:rice bran (DM basis). The negative aspect is that considerable amounts of non-protein-nitrogen (possibly as ammonium acetate/lactate) remain in the fermented substrate with unknown consequences when fed to pigs.

Experiment 2: Effect on the growth of pigs of replacing fish meal with fermented cassava pulp

This experiment aimed to evaluate the feeding of the protein-enriched cassava pulp-rice bran mixture as partial replacement for fish meal in a diet for growing-fattening pigs.

Materials and methods

Experimental design

Twenty-five crossbreed pigs with average initial body weight of 10 kg were housed in individual cages at the experimental farm of the Institute of Development Studies of Hue University of Agriculture and Forestry, Vietnam. The experiment was designed with five levels of replacement (0, 25, 50, 75 and 100%) of fish meal protein by protein from protein-enriched cassava pulp/rice bran in a basal diet of maize meal, rice bran and cassava root meal (Table 3). The pigs were allocated to 5 treatments with 3 replicates per treatment, each replicate with equal numbers of male and females. The treatments were:

CP0: All supplementary protein from fish meal (which accounted for 22.6% of dietary N in period 1 and 30.6% in period 2

CP25: 25% of fish meal protein replaced by crude protein from protein-enriched cassava pulp/rice bran (PECP)

CP50: 50% of fish meal protein replaced by crude protein from PECP

CP75: 75% of fish meal protein replaced by crude protein from PECP

CP100: 100% of fish meal protein replaced by crude protein from PECP

The diets were formulated to contain 186 and 157 g crude protein/kg DM in growth periods 1 and 2 (Tables 4 and 5). The PECP was produced following the procedure described un Experiment 1 for the 2% urea treatment, and was assumed to contain 27% of crude protein and 12.5% true protein based on the results of Experiment 1.

Table 3. Content of crude and true protein in diet ingredients (% in DM)

	Crude protein	True protein#
Maize	10	10
Cassava pulp	2.4	2.4
Rice bran	12	12
FM	60	60
SBM	46	46
PECP	27	12

PECP: Protein-enriched cassava pulp/rice bran
 # Assumed the same as crude protein except for true protein in PECP based on analysis in experiment 1

Table 4. Composition of diet ingredients (on DM basis except for DM which is on air-dry basis**)**

	DM	CP	EE	CF	Ash	Ca	P	Lysine	M
Fish meal	88	60	4.8	1.5	42.9	7.34	1.67	5.25	

Soybean meal	90	46	0	0	0	0	0	3.39
Maize	88.2	9.8	3.7	2	1.7	0.22	0.3	0.27
Cassava root meal	87.4	2.87	1.68	2.95	2.18	0.23	0.15	0.07
Rice bran	87.6	13	12.0	7.77	8.37	0.17	1.65	0.55
PECP	37	27	3.33	12	1.71	0.11	0.2	
Salt	85							
Premix	96							

Table 5. Ingredients (g/kg DM) in the diets

Ingredients	**CP0**	**CP25**	**CP50**	**CP75**	**CP100**
Period 15 – 50 kg (g/kg DM)					
Fish meal	70	52.5	35	17.5	0
Soybean meal	160	160	160	160	160
Maize	412	412	412	412	412
Cassava root meal	200	178.5	157	135.5	114
Rice bran	150	150	150	150	150

PECP	0	39	78	117	156
Salt	3	3	3	3	3
Premix	5	5	5	5	5
CP, g/kg DM	186	186	185	185	184
PECP, % of CP	0.0	5.7	11.4	17.1	22.9
PECP, % of FM	0.0	25.1	50.1	75.2	100
CP, g/kg#	186	175	164	153	142
Period 50 – 100 kg (g/kg DM)					
Fish meal	80	60	40	20	0
Soybean meal	80	80	80	80	80
Maize	390	390	390	390	390
Cassava root meal	242	217	193	168	144
Rice bran	200	200	200	200	200
PECP	0	45	89	134	178
Salt	3	3	3	3	3

Premix	5	5	5	5	5
CP, g/kg DM	157	157	156	155	155
PECP, % of CP	0.0	7.76	15.4	23.3	31.1
PECP, % of FM	0	25.3	50.1	75.4	100
CP, g/kg#	157	144	132	119	107

 # Excluding CP (crude protein) from PECP

Data recording

Feed offered and refused was recorded daily. The pigs were weighed every month.

Statistical analysis

Response trends in feed intake, growth rate and feed conversion (Y) were related to percent replacement of fish meal protein by PECR crude protein (X). Comparison of results for the zero and 25% fish meal protein replacement levels were by the GLM option in the ANOVA program of the Minitab (2016) software. Sources of variation were treatments (CPO versus CP25), replicates and error.

Results and discussion

The crude protein of the diets was in the range of 15 to 17% in DM in both periods (Table 6). By contrast, the content of "true" protein decreased linearly from 15 to 11% in DM as the fish meal was replaced by PECP. However, this analysis does not take account of the protein that could have been synthesized by bacteria in the pig intestine using recycled or dietary NPN as the source of ammonia (Colombus et al 2014). Thus the apparent reduction in true protein indicated by analysus of the feed may not be a true rrepresentation of the situation at the sites of metabolism.

Table 6. Mean values for crude and true protein the diets used in periods 1 and 2

	Protein in DM, %	
	Crude	**True**
Period 1		
CP0	15.5	14.5
CP25	17.2	14.4
CP50	15.6	14.2
CP75	15.9	11.9

CP100	15.1	11.5
Period 2		
CP0	15.6	14.7
CP25	16.9	13.7
CP50	16.1	13.5
CP75	15.1	13.0
CP100	15.2	11.3

Overall, there were linear depressions in DM intake and live weight gain as protein from PECP replaced that from fish meal (Table 7; Figures 5 and 6). There was no consistent trend in the data for DM feed conversion as fish meal protein was replaced by PECP protein (Figure 7).

Table 7. Mean values for changes in live weight, DM intake and DM feed conversion of pigs fed diets in which protein from protein-enriched cassava pulp replaced protein from fish

	PECP protein replacing fish meal protein, %					
	0	**25**	**50**	**75**	**100**	**SEM**
Init wt, kg	24.2	24.1	24.1	24.1	24.1	0.563
Final wt, kg	86.3	85.1	78.2	75.8	69.6	3.87
Daily gain, g/d	702	688	608	584	518	41.2
DM intake, g/d	1.98	1.75	1.75	1.86	1.43	0.126
DM conversion	2.82	2.53	2.93	3.19	2.81	0.182

Figure 5. Replacing fish meal with protein-enriched cassava pulp reduces linearly the DM feed intake

Figure 6. Replacing fish meal with protein-enriched cassava pulp reduces linearly the live weight gain

Figure 7. There was an indication of better feed conversion by replacing 25% of the fish meal protein w protein from protein-enriched cassava pulp but at higher levels of replacement feed conversion was wor

When the comparison of treatments was restricted to 0 and 25% replacement of fish meal protein (equivalent to raising the content of PECP from 0 and 4.5% of the diet DM), DM intake was reduced, live weight gain did not differ (*p=0.59*), and DM feed conversion tended (*p=0.08*) tended to be improved (Table 8).

Table 8. Effect of low level of PECP on DM intake, growth rate and feed conversion of growing-fattening pigs

	PECP, % in diet DM			
	0	**4.5**	**SEM**	***p***
LW gain, g/d	702	688	16.6	0.59
DMI, g/d	1.98	1.75	0.036	0.01
DM conv.	2.82	2.53	0.09	0.08

These results are broadly similar to those reported by Vanhnasin and Preston (2016) and Sengxayalth and Preston (2017b), namely that when fed as only a small proportion (4.5%) of the diet DM, PECP had marginal beneficial effects on pig performance but at higher levels there was a marked deterioration in both growth rate and feed conversion.

As only about 50% of PECP nitrogen was present as true protein (Experiment 1), the complete replacement of fish meal by PECP meant that the NPN component of the diet increased to approximately 8 g N/kg of diet DM. If this NPN was in the form of NH_4^+-N, it poses the question: was this sufficient to cause sub-acute ammonia toxicity? And if this was so, is this the explanation for the deterioration in growth performance as the proportion of PECP in the diet was increased from 4.5 to 15-17% of diet DM?

Conclusions

- After 7 days of fermenting a 70:30 mixture of cassava pulp and rice bran with urea (from 0 to

2% in DM) and DAP (1% in DM) and yeast, the level of true protein reached a range of 12.3 to 12.5% in DM when urea levels were from 0.5 to 2% of the substrate DM. Comparable values for crude protein were in the range 24 to 27.5% in DM. These increases in concentrations of “true” and “crude” protein were associated with a loss of some 30% of the substrate DM indicating that approximately 3 kg of substrate DM were fermented to produce 1 kg of yeast protein, Another explanation for the increase in "crude" protein could have been N-fixation from the air as *Pichia pastoris* is thought to have this capacity.

- With 25% fish meal protein replacement (4% PECP in the diet DM), in a mixed diet of maize, rice bran, fish meal and soybean meal fed to growing-fattening pigs, feed intake was reduced, live weight gain did not differ, and DM feed conversion tended to be improved. At higher proportions of PECP in the diet (6.8 to 16.5%), there was a linear depression in live weight gain (overall reduction of 25%). It is suggested that the reduction in live weight gain with more than 4.5% PECP in the diet DM may have been caused by sub-acute toxicity caused by residual NPN compounds in the PECP because of incomplete conversion of the NPN to yeast protein.

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