SLURRY AND MANURE AS A SOURCE OF BIOETHANOL FOR SUSTAINABLE MOBILITY IN RURAL AREAS

Daniela Bona[1], Alessia Vecchiet[2], Silvia Silvestri[1], Flavio Fornasier[3], Raffaele Guzzon[1]
[1] Edmund Mach Foundation, Via E. Mach, 1 38010 San Michele a/A (TN) Italy
tel. 0039 0461 615383, fax. 0039 0461 615313, e-mail: daniela.bona@iasma.it
[2] Centre for Theoretical and Applied Ecology, Via Licinio 44 Gorizia (GO) Italy
[3] National Council for Agricultural Research (CRA-RPS), Via Trieste, 23 Gorizia (GO) Italy

ABSTRACT: This work reports part of the activity of the project **ZOOTANOLO "The production of bioethanol as an innovative energy use of manure".** The first phase was dedicated to verify at lab scale the feasibility of using manure to produce bioethanol. Manure have good content of carbohydrate (cellulose, hemicellulose, particularly); scope of the work is the enhancement of this unused lignocellulosic fraction. The experimental and technical activity divides into several subsequently steps: pre-treatment of the animal biomass, hydrolysis of molecules of cellulose, hemicelluloses and starch by enzymes; fermentation by yeast (*Saccharomyces cerevisiae* and *Pichia stipitis*) and ethanol distillation. Related topics under study are the application of immobilization techniques to overcome inhibition phenomena and treatments on the distillation waste, aimed at nitrogen removal and at the evaluation of residual potential energy. The data obtained will be used to energetic, technical, economic and environmental evaluation and feasibility study, with the final aim to contribute to the development of the production chain of second generation bioethanol in rural areas.
Keywords: manure, lignocellulosics biomass, manure pre-treatment, enzymatic and acid hydrolysis, fermentation

1 INTRODUCTION

The work will provide innovative possibility in three areas, responding to the need to develop new and sustainable initiatives for the start of the bioethanol sector, the protection of groundwater from leaching of nitrates from livestock and eliminate the competition between the use of food and non – food agricultural crops and land.

The replacement of fossil fuels with biofuels, and bio-ethanol in particular, is one of the strategies adopted by the European Commission and the Italian State to combat climate change resulting from the accumulation of greenhouse gases in the atmosphere; at this time the national production of ethanol is inadequacy.

Moreover the sustainable management of manure and slurry is in some cases difficult, because of intensive production in restricted areas.

Anaerobic digestion is currently the most common solution for management and energy production from these material, but it seems important to investigate the energetic, economic and environmental sustainability of different options. Developing an economical process for bioethanol production from manure it would also provide alternative used for this material treatment and would raise farmer's income while decreasing at the same time the GHG emission.

1.1 Manure to development 2nd generation bioethanol

Animal manure contain lignocelluloses, polysaccharides, proteins and other compounds, and the ability to convert these material into bioethanol appears an innovative perspective [1]. Dairy manure, for example, contains 12 % of hemicellulose and 22 % of cellulose (by weight) and represents a large potential source of carbohydrates such as xylose and glucose [2].

Pig slurry contains 53.8 % carbohydrates on total solids, hens slurry and broilers manure have 16-18 % of hemicellulose and 8-13 % cellulose, expressed on dry matter (% dm).

However cattle manure is a special fibrous material, with nitrogen content of around 2,5 %, much higher than other lignocellulosic biomass (1 % wheat straw). This chemical composition enables more easily some side-reaction such as browing reaction and dehydration that influence the final sugar yield [1] .

Previous studies on the hydrolysis of manure have showed that the optimal conditions were 650 FPU/L cellulase 250 IU/L β-glucosidase and 50 g/l substrate at pH 4.8 and 46 °C. The glucose conversion yield under the optimal conditions reached 52 %. Enzymatic hydrolysis appears a promising method, since it is environmentally friendly and moderate. However the comparison with chemical hydrolysis showed that decrystallization is the critical step when trying to improve glucose yield [1] .

1.2 The ZOOTANOLO project

This work is part of the project ZOOTANOLO " *The production of bioethanol as an innovative energy use of manure*", co-funded by the Italian Ministry of Agriculture. The total project duration is three years; the first year just ended.

The aim is the exploitation of lignocellulosic fraction for the production of biofuels, and the analysis of data obtained by experimental activity to assess the economic, environmental and energetic feasibility of this solution.

The experimental plan divides in several steps: characterization of different kinds of manure, chemical and mechanical pre-treatment, enzymatic hydrolysis, fermentation by yeast and process yield calculation, application of innovative techniques of cellular immobilization and management of distillation waste by anaerobic digestion and by systems for nitrogen removal.

The results presented are from the firsts year of the project.

Rural areas considered are within Friuli-Venezia Giulia and Trentino. Cattle, heifers and poultry manure were tested in the first year, because more representative of local livestock (poultry in Friuli and cattle in Trentino).

2 MATERIAL AND METHODS

2.1 Sampling, characterization and pre-treatment

Samples of different manure were collected in different farms, then they were characterized and stored at - 20°C.

Preliminary analyses included total solids (TS), moisture content, total volatile solids (TVS).

The content of cellulose, hemicellulose and lignin in manure was determined by the Van Soest procedure, i.e. by the analysis of NDF, ADF and ADL fraction. NDF is usually used to estimate the total lignocellulosic material (including cellulose, hemicellulose and lignin); ADF is used to estimate the content of lignin and cellulose. Hemicellulose content can be determined by the difference between them (% NDF - % ADF). The material was first milled, washed and filtered on a 200 micron sieve, then dried at 105 °C. This pre-treatment was necessary to remove completely some components and to avoid consequently the very dark coloration of material after the treatment with detergent.

Other analyses for a complete characterization include total carbon, total nitrogen, nitrates, ammonia and other nutrient for agronomic and environmental assessment on the fresh manure and the waste after each phase of the process.

The determination of total soluble sugars was carried out by reaction with anthrone after centrifugation at 20,000 x g for 5 minutes. Fermentable sugar are expressed as glucose equivalent (g/l).

2.2 Pre-treatment

Because of the high content of nitrogen in the sample and the complexity of the matrix, different systems of pre-treatment were considered.

In order to remove nutrients the manure was separated into liquid and solid fraction by centrifugation (3000 rpm at 20 min, after dilution 1:2 in weight) and filtration with sieve and filter paper.

Simultaneously different procedures of washing were performed: subsequent cycles of dilution and liquid fraction removal.

Before further processing the samples were diluted with water (up to about 30 – 50 g/l dm) and homogenized.

2.3 Hydrolysis

The enzymatic hydrolysis was performed on the fresh manure and washed manure.

Commercial enzymes were utilized: Amylyve TC (α-amylase) from *Aspergillus niger* with high thermal stability (up to 75 °C) even at low pH values that are suitable for apple juice treatment; Filterlyve TC (fungal β-glucanase) from selected strain of *Aspergillus niger*; Cellulyve (1-4-β-endo-D-glucanase, cellobiohydrolase and cellobiase) from *Trichoderma reesei;* Lyvanol Devisco, hemicelluase preparation from *Trichoderma longibrachiatum*.

The hydrolysis was performed in a bioreactor with a capacity of 2 l. The trials were prepared with 1 l of sample, pH value correct to 4.5 and 180 rpm mixing.

The reaction is divided into two steps: a first one with α-amylase and β-glucanase for 2 h at 55 °C; a second step with cellulase and hemicellulase for 72 h at 50 °C.

Acid hydrolysis, introduced to improve sugar yield in the second phase of the work, based on literature reference [3, 2, 4]. Thus the samples are divided into 1000 ml bottles and treated with sulphuric acid at a concentration of 5 % in 1:5 ratio w/w and then heated in autoclave for 20 min at 121 °C. The sample was filtered: liquid fraction has been preserved for chemical analysis and subsequent fermentation, while solid fraction was diluted (to the substrate concentration values between 30 and 50 g/l of dry matter) and was trial by enzymes as has been done in the first series of tests.

To verify the best conditions and the possible effect of inhibition, were made other trials of acid hydrolysis with different acid concentration (2 % w/w or 3 % w/w) and heating system (121 °C for 5 min; 110° for 60 min in laboratory stove). Further to try out was also tested the direct fermentation of the whole samples (without solid-liquid separation), correcting pH to 4.5 – 5.0.

2.4 Fermentation by yeast

Microorganisms used belong to the ARS Culture collection (NRRL). Fermentation tests were performed in Yeast Medium broth (Yeast Extract 3 g/l, Malt Extract 3 g/l, Peptone 5 g/l), all component purchased by OXOID; pH 6.2. The temperature set was 25 °C – 30 °C. Yeast inoculums was adjusted to a final density of 106 cell/ml). The evolution of alcoholic fermentations was followed by measuring the alcohol determination by enzymatic kit based on the alcohol dehydrogenase and aldehyde dehydrogenase followed by measurement of NADH absorbance (340 nm).

3 FIRST RESULTS

3.1 Distribution at local and national level

Poultry farming in Friuli-Venezia Giulia is consistent: 332 farms divided in 108 broiler, 231 hens, 77 other (ISATA, 2007). In some cases there are multiple forms breeding in the same farm, resulting in overlap of data. The total number of animals in the region is 5,234,581; in Italy there are 80,325 poultry farms with a 157,346,105 animals.

Three different kind of samples were considered: manure of hens without litter, poultry manure of broiler with litter (straw) and poultry manure of broilers with other kind of litter (wood chips).

In the Trento Province, dairy cattle farms are prevalent. The data collected on 2009, converted to LSU (Adult Cattle Unit) show a total consistency of 34.657 LU, distributed in 1,500 farms. The average LU is 26 LU/farm; therefore the size of farms is mainly medium-small (mountain livestock). The average production of dry matter excreted amounts to 8.05 kg/head per day in intensive livestock areas (28 l / day of milk) and 7.7 kg/head per day in less specialized farms. Regarding the litter, 1 kg/day per head of straw for slurry and 3 kg/day per head for manure can be the amount considered.

The national situation of the livestock breeding is obviously different with more specialized farms, usually bigger than those in mountain areas. The data ISTAT show in Italy 124,000 cattle farms (59.2 % of total farms) with a corresponding number of animals 5.7 million of cattle. More than half of the farms are concentrated in Northern Italy.

Four different samples were considered: dairy slurry without litter, heifers slurry (without litter), dairy manure with litter (straw) and heifers manure with litter. The farms using sawdust as litter are relatively few, therefore not considered in the present study.

3.2 Characterization and pre-treatment of biomass

Samples were chosen among the whole samples analyzed are reported in Table 1.

Table I: Collected samples of manure and slurry

Manure and Slurry	TS %	TVS %dm	Ash %dm
Dairy manure	15	78.40	21.60
Dairy slurry	11	83.96	16.04
Heifers manure	21	81.69	18.31
Heifers slurry	10	81.47	18.53
Poultry manure	23	61.40	38.60
Poultry manure (straw)	31	66.67	33.33
Poultry manure (sawdust)	58	89.00	11.00

Each sample was also centrifuged analyzing the solid part, even if the final decision was to use the two whole sample types, not separating the liquid fraction.

In the second phase of the experimental activity was also considered the straw used for bedding, in order to define the contribution of the litter fiber than the undigested fiber.

Preliminary results showed a good content of cellulose and hemicellulose in sample manures. The determination of starch content in the samples was very difficult. Figure 1 shows the composition of dry matter fiber (cellulose, hemicellulose, lignin and ash).

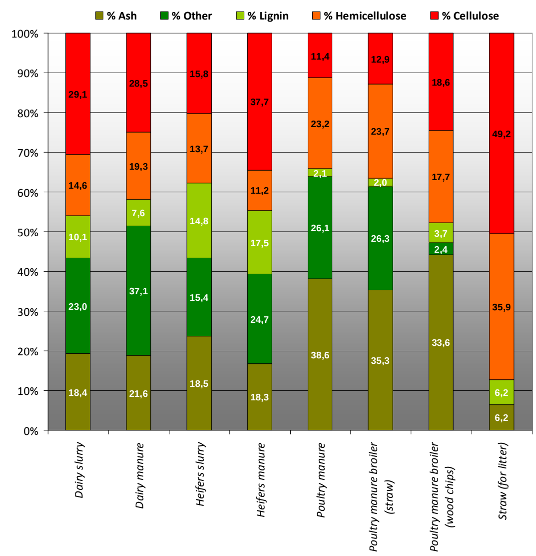

Figure 1: Dry matter composition of animal waste

To note that considerable amount of cellulose and hemicellulose in the samples is due to the straw as bedding.

Cow manure and slurry seem very promising in relation to the content of hemicellulose and cellulose. On the contrary addressing poultry manure to this productive processes is very difficult due to high content of nitrogen and the high presence of ash.

The content of fiber expressed in g/kg is: 30 – 35 g/kg cellulose, 18 – 19 g/kg hemicellulose and 7 – 9 g/kg lignin for dairy manure; 29 g/kg cellulose, 14.6 g/kg hemicellulose, 10 g/kg lignin for dairy slurry; about 15 g/kg cellulose, 13.7 g/kg hemicellulose, 14.82 g/kg lignin for dairy manure. On the other hand poultry manure show best content of cellulose (between 26 and 56 g/kg in relation to straw or sawdust as litter), hemicellulose (53 – 54 g/kg) and a lower content of lignin (between 4 and 10 g/kg in relation to straw or sawdust as litter), but also the ash content is high. To use these is necessary to make a mechanical pre-treated. All the sample are been diluted and milled before the process. From different trials has been identified the best dilution of the samples. Indeed the dilution up to a final content of 30 – 50 g/l of dry matter is fundamental to be able to proceed with hydrolysis and fermentation.

3.3 Hydrolysis

The enzymatic hydrolyses were carried out on both samples washed and centrifuged and samples simply diluted. There were no differences in the yields of fermentable sugars. The comparison between washed and unwashed samples did not show significant differences on the content of polysaccharides and fermentable sugar. Even the pre-treatment (washing or separation) demonstrated not strictly necessary.

The sugars released from poultry manure amounted to 27 g/kg dm for the samples without litter and straw, while the sample with a litter of sawdust release 50 g/kg dm of sugars. The hydrolytic yields were very similar for all sample treated (i.e. all values of around 7.0 to 7.5%).

For cow manure the sugar determinations showed a better performance of dairy slurry and dairy manure (42.24 g/kg dm manure, 21.82 g/kg dm slurry). The hydrolytic yields is 6.78 % and 4.97 % respectively. Unlike the heifers manure have not greater yielded (8.34 g/kg dm of sugar) despite a greater amount of cellulose in fresh sample (37.25 % on dry weight). Heifers slurry have a good concentration of fermentable sugar (29.24 g/kg dm), but very low hydrolytic yield.

The results of first tests are reported in table II.

Tab II: Hydrolytic yield of first samples (enzymatic hydrolysis)

Sample	Sugar g/kg dm	Yield %
Dairy manure	32.40	6.78
Dairy slurry	14.66	4.97
Heifers manure	2.50	0.44
Heifers slurry	4.78	0.87
Poultry manure hens	26.96	7.35
Poultry manure broiler (straw)	26.70	7.37
Poultry manure broiler (sawdust)	49.10	7.14

Therefore in the second experimental step, was introduced on the same samples, a weak acid hydrolysis, before the enzymatic hydrolysis. This works with dilute sulphuric acid (5 % w/w) which aims to improve the fiber decrystallization useful for the following enzymatic hydrolysis and make a first hydrolysis of cellulose and hemicellulose. This treatment was applied to straw, cattle manure and slurry (without bedding) and from poultry.

Acid hydrolysis has produced 53.25 g/kg dm and 90.48 g/kg dm of sugar obtained for both test on the same sample (poultry manure with straw as litter). Correspond yields are 14.64 % and 24.88 % respectively. The subsequent enzymatic hydrolysis gave a value similar than acid hydrolysis (i.e. 15.0 g/l of sugars).

The acid hydrolysis on cattle manure produced 47.92 g/kg dm (16.24 % yield) by dairy slurry, 138.23 g/kg dm (yield 28.24 %) by the dairy manure and finally 83.13 g/kg dm (yield 13.19 %) by only straw. The new enzymatic hydrolysis performed on the solid phase of acid sample increased the amount of total sugars (the liquid fraction containing all the sugars produced in the first steps were removed). Thus were produced more 49.75 g/kg dm (yield 16.86 %) by dairy slurry, 146.39 g/kg dm (yield 30.63 %) by dairy manure and then 147.85 g/kg dm (yield 23.47 %) by only straw.

The results of acid and enzymatic hydrolysis are reported in table II and figures 2 and 3.

Tab II: Hydrolytic yield of second step of experimental activity (acid hydrolysis and enzymatic hydrolysis)

Sample	Acid *H.* yield %	Enzymatic *H* . yield %
Dairy manure	28.24	30.63
Dairy slurry	16.24	16.86
Heifers slurry	13.19	23.47
Poultry manure broiler (straw)	24.88	8.75

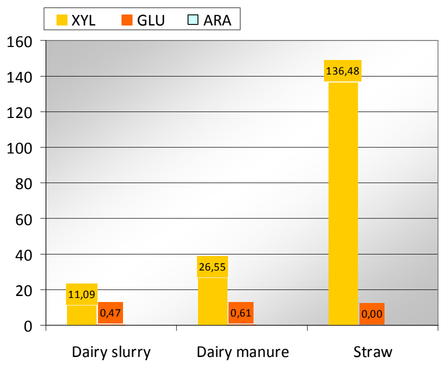

Figure 2: Different production of pentose and hexose by acid hydrolysis. XIL: xylose; GLU: glucose; ARA: arabinose.

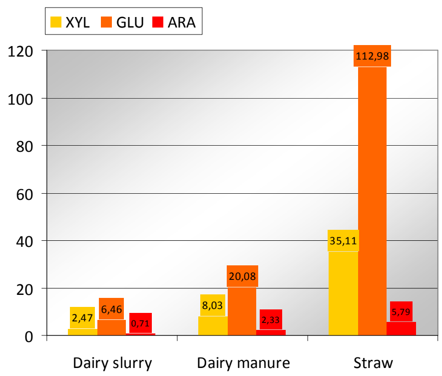

Figure 3: Different production of pentose and hexose by enzymatic hydrolysis (after acid treatment). XIL: xylose; GLU: glucose; ARA: arabinose.

Similar results were obtained for samples treated with lower concentrations of sulphuric acid and different temperatures. The treatment which gives the best results at the moment (highest concentration of fermentable sugar) is the treatment with acid at 3 % and heating at 121 ° C for 5 min in autoclave.

This tests are performed on two samples: dairy manure and straw. The results obtained are presented in the figure 4.

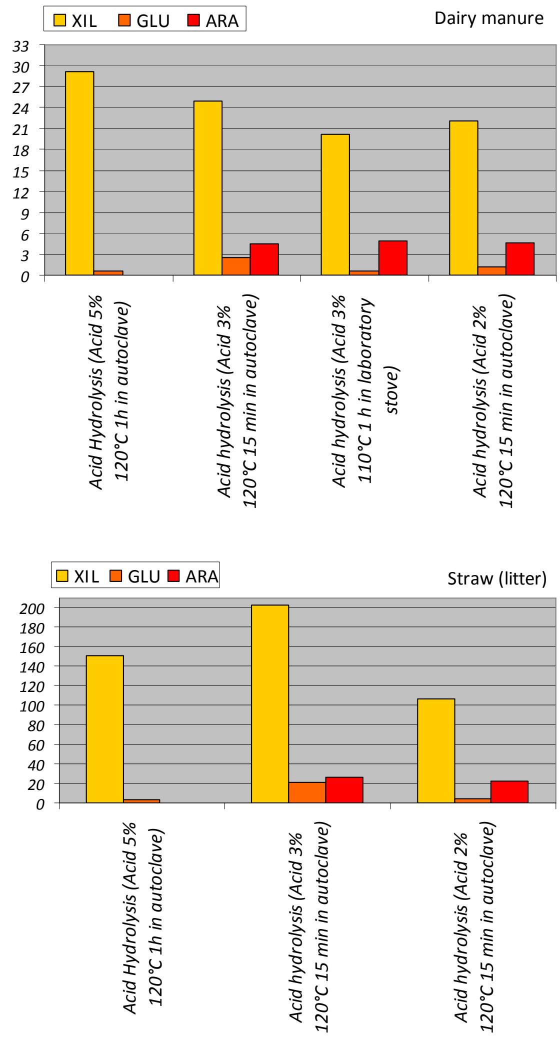

Figure 4: Comparison between different acid hydrolysis performed on the two samples: dairy manure and straw to define the best conditions. XIL: xylose; GLU: glucose; ARA: arabinose.

3.3 Fermentation

Given the low production of fermentable sugars in the hydrolysis, fermentation tests were prepared using the selected yeasts employed in the experimental activity of the project (*Saccharomyces cerevisiae* and *Pichia stipitis*)

Dairy slurry was added with known content (0 g/l - 5 g/l – 10 g/l – 20 g/l – 30 g/l) of glucose for *S. cerevisiae* fermentation and xylose for *P. stipits* fermentation.

The tests allowed to define the minimum concentration for a good yield*:* 10 g/l for *S. cerevisiae*; while the *P. stipis* yield was very low. Further tests will be set up to optimize performance at low concentration of pentose sugar. *Pichia stipitis* indeed exhibits high ethanol production in presence of high content of xylose [5] .

Fermentation tests were performed on most promising samples in terms of fermentable sugar: poultry manure without litter, poultry manure with straw, poultry manure with sawdust as litter, dairy manure with straw as litter, heifers slurry (without litter), and dairy slurry (without litter).

All samples were fermented hydrolyzed sample and fresh sample (as control).

The fermentation yields obtained are 5.15 % by the hydrolyzed heifers slurry, 5.56 % by hydrolyzed dairy slurry and 7.80 % by hydrolyzed dairy manure (corresponding to 10.03 % and then 10.81 % and 15.18 % compared to the theoretical yield).

For the poultry hydrolyzed sample, fermentation yield is much better with respect to the control: 0.33 % (fresh manure) and 17.32 % (after enzymatic hydrolysis). Sample with sawdust as litter give a results under verify (55 -58 %).

Generally the fermentation yields do not show great differences between samples hydrolyzed and not hydrolyzed (as show in table III). This is probably due to the low content of fermentable sugars produced by the enzymatic hydrolysis.

Tab III: Fermentative yield of samples

Sample	Fermentation yield[1] %	Fermentation yield[2]
Dairy manure	7.80	15.18
Dairy manure (c)	4.72	9.18
Dairy slurry	5.56	10.81
Dairy slurry (c)	8.00	15.56
Heifers slurry	5.15	10.03
Heifers slurry (c)	3.03	5.90
Poultry m. hens	3.33	6.47
Poultry m. hens (c)	20.90*	40.60*
Poultry m. broiler (straw)	8.90	17.32
Poultry m. broiler (straw) (c)	0.17	0.33
Poultry m. broiler (sawdust)	58.50*	55.45*
Poultry m. br. (sawdust) (c)	29.73*	57.80*

1 Fermentative yield obtained
2 Fermentative yield in relation to theoretical fermentative yield (51,4 %)
* *values to be confirmed*
(c) *control*

Most promising results are expected from fermentation of samples after acid and enzymatic hydrolysis (tests are in progress). At the moment was fermented only the sample of poultry manure (with straw as litter). The preliminary results show the improvement of the fermentation yield: 23.20 % in sample after acid and enzymatic hydrolysis, but the yield remains low in the sample after only acid hydrolysis.

The determination of list glucose, xylose concentration instead of glucose equivalent allows to calculate the exactly enzymes performance and fermentation yield for *Pichia stipitis* and *Saccharomyces cerevisiae*.

The presented results are preliminary; the tests in progress are aimed to confirm and enhance the data achieved so far.

4 CONCLUSIONS AND PERSPECTIVES

The first data here reported show that livestock manure can be a good matrix for the production of ethanol, with up to 35% of hydrolysable fiber detected.

The minimum sugar content in the samples to enable a good fermentation must be at least 15 g/l. It has to be better investigated the performance of *Pichia stipitis* at lower concentrations of sugar. It is very important to investigate yields of *Pichia stipitis* in animal manure, seen that the xylose concentration is good.

It is evident from the tests made that acid hydrolysis before the enzymatic one is necessary to fiber degradation. The fermentation data (of latest sample) will be important to improve the current production obtained.

Interesting opportunities addressed during the second year of activity will deal with the application of immobilization techniques to improve the efficiency of fermentation and the replacement of commercial cellulase enzymes with the fungi *Trichoderma reseei*, as the manure should be a suitable substrate for cellulase production by *T. reseei* [6].

Another important issue concerns the development of solutions for final treatment of distillation waste. Therefore the biomethane potential and the co-digestion with other biomass will be tested.

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6 LOGO SPACE

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