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Prospecting for sludge bacteria from a poultry slaughterhouse, with potential for degrading organic substances

Prospecção de bactérias de lodo de abatedouro de aves com potencial degradador de substâncias orgânicas

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Abstract

The sludge produced in wastewater treatment plants of slaughterhouses is a rich source of chemical, organic, and microbiological constituents that can be biotechnologically exploited. The purpose of this study was to (i) conduct a chemical analysis of the sludge, and (ii) isolate, quantify, and describe the amylolytic, cellulolytic, ligninolytic, proteolytic, and keratinolytic bacteria in the sludge. Sludge samples were collected at the wastewater treatment plant of the Francap SA poultry company. The nutrient contents, C/N ratio, and pH were determined. For the bacterial count, 10 g sludge was diluted in 90 mL saline solution, which was serially diluted to 10^{-12}. Aliquots of 100 µL of each dilution were transferred to selective media for isolation of bacteria that degrade organic substances. The colony-forming units were determined for each culture medium. Individual colonies were purified and characterized morphologically. The sludge contained 9.5, 1.21, and 0.45 dag kg^{-1} of N, P, and K, respectively. Fifty-two isolates were purified and characterized, with 2.11×10^{12} to 9.55×10^{15} colony-forming units per g sludge. In conclusion, the sludge generated in poultry slaughterhouse wastewater treatment plants is a rich source of organo-mineral constituents and bacteria with biotechnological potential for degrading organic substances.

Key words: Agricultural microbiology. Organic matter. Solid waste.

Resumo

Os lodos produzidos nas estações de tratamento de efluentes de abatedouros são uma fonte de riqueza química, orgânica e microbiológica que precisa ser explorada biotecnologicamente. Objetivou-se neste estudo: (i) realizar a caracterização química do lodo e (ii) isolar, quantificar e caracterizar bactérias amilolíticas, celulolíticas, ligninolíticas, proteolíticas e queratinolíticas do lodo. Amostras de lodo foram coletadas na estação de tratamento de efluentes da empresa avícola FRANCAP S.A. Foram determinados os teores de nutrientes, a relação C/N e o pH. Para a quantificação bacteriana, 10 g de lodo foram diluídos em 90 mL de solução salina e dela, realizaram-se diluições seriadas até 10^{-12}. Alíquotas de 100 µL de cada diluição foram transferidas aos meios seletivos para isolamento de bactérias degradadoras de substâncias orgânicas. Determinaram-se as unidades formadoras de colônia para cada meio de cultivo. Colônias individuais foram purificadas e caracterizadas morfologicamente. O lodo apresentou teores de N, P e K, de 9,5, 1,21 e 0,45 dag kg^{-1}, respectivamente. Foram purificados e caracterizados 52 isolados, as unidades formadoras de colônia variaram entre $2,11 \times 10^{12}$ a $9,55 \times 10^{15}$. Conclui-se que os lodos gerados nas estações de tratamento de efluentes de abatedouro de aves são uma fonte de riqueza organo-mineral, além de apresentar bactérias com potencial biotecnológico de degradar substâncias orgânicas.

Palavras-chave: Matéria orgânica. Microbiologia agrícola. Resíduos sólidos.

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Introduction

In 2013, Brazil produced 12.3 million tons of poultry, of which 69% was used locally and 31% was used in the international market (UBABEF, 2014). The state of Minas Gerais accounts for 8% of the total output and ranks fourth in poultry production. Despite the existence of many poultry farms, only 300 are registered in the State (IMA, 2013), hampering the control of the processes and management of by-products of this agricultural industry.

One of the by-products generated in the poultry industry is sludge from treatment of slaughterhouse effluent, defined as a mixture of substances consisting of mineral colloids, particles, and decomposed organic matter in aqueous medium (CONAMA, 2006).

If untreated, the disposal of such sludge leads to water and soil contamination, odor, and increased incidence of diseases (HECK et al., 2012). To avoid these problems, the material is transferred to landfills, incinerated, recycled, composted, or transformed into biofertilizers (HECK et al., 2012). For application in agriculture, the sludge should be analyzed for agronomic potential, and for toxic substances and pathogens. The presence of viable helminth eggs, salmonella, and enteric viruses should also be assessed (CONAMA, 2006), with special consideration to heat-tolerant coliforms.

Apart from pathogenic microorganisms, sludge can potentially harbor non-pathogenic microorganisms, such as bacteria that degrade organic substances. Among these are: (i) amylolytic bacteria that convert starch into fatty acids (GABARRA, 2001), (ii) cellulolytic bacteria that degrade polymeric carbohydrates (PIRES, 2008), (iii) ligninolytic bacteria that use lignin as a carbon source (BASTOS-NETO, 2012), (iv) proteolytic bacteria that degrade proteins (CARPINÉ et al., 2010), and (v) keratinolytic bacteria that decompose keratinized structures (PACHECO, 2013).

In this context, biotechnology research is needed to focus on the selection of bacteria able to transform and reduce organic by-products, hence mitigating environmental impacts.

The purpose of this study was to (i) chemically characterize the sludge generated in the wastewater treatment plant of a poultry slaughterhouse, and (ii) isolate, quantify, and characterize bacteria, with potential for degrading organic matter, from the sludge.

Material and Methods

Collecting sludge samples

Sludge samples were collected from the wastewater treatment system of the Activated Sludge/Extended Aeration type at a poultry slaughterhouse of FRANCAP S.A., in the city of Pará de Minas, MG (FRANCAP, 2015). Samples were transported to the floriculture sector of the Universidade Federal de Viçosa – Campus Florestal, where microbiological analyses were carried out in 2013 and 2014.

Chemical analysis of the sludge

Sludge samples were dried in a forced circulation oven at 60 °C and sent to the Soil Analysis Laboratory Viçosa Ltd. to determine the total contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), boron (B), and cobalt (Co), as well as the percentage of organic carbon (C), C/N ratio, and pH (EMBRAPA, 1997).

Isolation and quantification of bacteria degrading organic matter

Sludge samples (10 g) were resuspended in 90 mL saline solution (NaCl, 0.85 g L^{-1}), and diluted serially in 10-fold steps to 10^{-12}.

For the isolation of amylolytic bacteria, 100-µL aliquots of each dilution were transferred to Petri dishes containing solid medium, containing the

following (per liter of distilled water): 10 g starch, 0.4 g ammonium chloride (NH_4Cl), 0.8 g dibasic potassium phosphate (K_2HPO_4), 0.12 g calcium chloride ($CaCl_2.2H_2O$), 0.12 g magnesium sulfate ($MgSO_4.7H_2O$) 15 g bacteriological agar, and 2 mL of the fungicide Cerconil (0.1 g L^{-1}). The pH was adjusted to 6.5 with sulfuric acid (H_2SO_4) and/or potassium hydroxide (KOH). The plates were incubated at 30 °C for 3 days. After growth of the colonies, staining was performed with lugol (potassium iodide, KI, 4%); the formation of a translucent halo around the colony indicated amylase activity (BASTOS-NETO et al., 2012).

For the isolation of cellulolytic bacteria, samples of 100 µL of each dilution were transferred to Petri dishes with solid medium, containing the following (per liter of distilled water): 10 g microcrystalline cellulose (MCC), 3 g sodium nitrate ($NaNO_3$), 1 g ammonium sulfate [$(NH_4)_2SO_4$], 0.5 g $MgSO_4$, 0.5 g potassium chloride (KCl), 10 g iron sulfate heptahydrate ($FeSO_4.7H_2O$), 20 g bacteriological agar, and 2 mL Cerconil (0.1 g L^{-1}), with the pH adjusted to 6.5 with H_2SO_4 and/or KOH. After incubation at 30 °C for 3 days, 10 mL Congo red dye (2.5 g L^{-1}) in 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0, was added to the plates for 30 min, followed by washing with 5 mL NaCl (0.5 mmol L^{-1}) (BORTOLAZZO, 2011).

For the isolation of ligninolytic bacteria, 100 µL samples per dilution were transferred to Petri dishes with solid medium, containing the following (per liter of distilled water): 1.2 g $NaNO_3$, 3.0 g monobasic potassium phosphate (KH_2PO_4), 6.0 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2.2H_2O$, 0.001g zinc sulfate ($ZnSO_4.7H_2O$), 0.01 g manganese sulfate ($MnSO_4.7H_2O$), 1.0 g yeast extract, 10.0 g lignin, 15 g bacteriological agar, and 2 mL Cerconil (0.1 g L^{-1}), with the pH adjusted to 6.5 with H_2SO_4 and/or KOH. The dishes were incubated at 30 °C for 3 days (MARTINEZ, 2009).

For the isolation of proteolytic bacteria, 100 µL samples per dilution were transferred to Petri dishes with solid medium, containing the following (per liter of distilled water): 100 mL skimmed milk, 5 g meat peptone, 3 g yeast extract, 12 g bacteriological agar, and 4 mL Cerconil (0.1 g L^{-1}), with the pH adjusted to 6.5 with H_2SO_4 and/or KOH. The dishes were incubated at 30 °C for 3 days (BACH, 2010).

To isolate keratinolytic bacteria, 100 µL samples per dilution were placed on Petri dishes with solid medium, containing the following (per liter of distilled water): 1 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 15 g bacteriological agar, and 2 mL Cerconil (0.1 g L^{-1}), with the pH adjusted to 6.5 with H_2SO_4 and/or KOH. The feather meal was prepared by soaking the feathers in 1% Triton X-100 detergent for 24 h, followed by washing in distilled water, drying at 60 °C, and grinding in a Wiley mill (BACH, 2010). The plates were incubated at 30 °C for 3 days.

For all culture media, the bacteria were quantified by counting the colony-forming units (CFU).

For purification of bacterial isolates, the individual colonies from the highest dilutions were transferred from the culture media to liquid DYGS medium (2 g glucose, 2 g malic acid, 1.5 g bacteriological peptone, 2 g yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, and 1.5 g glutamic acid in 1000 mL distilled water, adjusted to pH 6.8) and maintained at 30 °C and 120 rpm for 24 h. The bacteria were then transferred to Petri dishes containing the same growth medium to check the purity of the isolates. Isolates were stored in plastic microtubes containing autoclaved distilled water for routine use.

Morphological characterization of bacterial isolates

All bacterial isolates were analyzed based on the characteristics of colonies and cells. After 5 days of growth on solid DYGS medium, bacterial colonies were characterized for their size, shape (round or irregular), top (flat, lens, convex, pulvinate, umbonate, umbilicated), edge (undulated, lobed, indented, filamentous, integer), and surface (flat, rough, papillate), as proposed by Perin (2002). The cells were described based on the variable cell shape

and Gram staining. The Gram test involved treating a bacterial smear on a slide successively with crystal violet reagent for 1 min, lugol for 1 min, alcohol for 5 s, and safranin for 30 s. Thereafter, the slide was examined under an optical microscope. Bacteria that stained blue-violet were characterized as gram-positive and those that stained red as gram-negative.

Statistical analysis

The CFU data were log-transformed for subsequent calculation of the mean and mean standard error for each medium, with three replications.

Results and Discussion

Chemical sludge analysis

The values in Table 1 and several previous studies (COSTA et al., 2008; SOUSA NETO et al., 2012; RIBEIRO et al., 2012; SANTOS et al., 2014) showed that the sludge is a rich chemical and organic source and can be exploited for different uses. According to Resolution No. 375 of CONAMA of August 29, 2006, the sludge can be used as biofertilizer for crops that are not pasture or vegetable species, tubers, and flooded crops or for other crops whose edible portion is in contact with the soil. It can also be used in substrates, as soil conditioners and, as demonstrated in this study, as an initial source for the isolation of bacteria with biotechnological potential.

Quantification and isolation of bacteria degrading organic substances

Table 2 shows the numbers of aerobic bacterial isolates with the potential to degrade different organic compounds through selective culture, the respective dilution, and quantification of CFU.

Table 1. Chemical analysis of sludge from the activated sludge/extended aeration treatment station of the company FRANCAP S.A.

N	P	K	Ca	Mg	S	C	Zn	Fe	Mn	Cu	B	pH	C/N
----------dag/kg----------							----------mg/kg----------					($H_2$0)	
9.5	1.21	0.45	1.35	0.18	0.77	18.72	517	1908	114	342	8.3	6.7	1.96

Total contents, determined in acid extract (nitric acid with perchloric acid)
N – Kjeldahl method
C – Walkley-Black method.

Table 2. Number of bacterial isolates through selective culturing, the respective dilution, and quantification of colony-forming units (CFU).

Culture medium	Number of isolates	Dilution	CFU (log CFU/g sludge)
Amylolytic	10	10^{-9}	12.8
Cellulolytic	2	10^{-12}	16.0
Keratinolytic	15	10^{-8}	13.5
Ligninolytic	10	10^{-10}	14.0
Proteolytic	15	10^{-8}	12.0

These results reflect the type of wastewater management used by the company FRANCAP S.A., which is activated sludge with extended aeration. The activated sludge system used in the wastewater treatment is a process in which a biological mass grows and flocculates as long as oxygen is present, thus permitting the proliferation of aerobic microorganisms (SOBRINHO, 1983). In this regard, 52 morphotypes of aerobic bacteria were isolated from the five selective media for organic matter-degrading microbes, since 95% of the microbial population present in the organic matter belongs to the group of decomposing and, mainly, heterotrophic bacteria, responsible for the biodegradation of the soluble organic fraction of the matrix (FERREIRA et al., 2006).

The number of amylolytic bacterial isolates, (10; Table 2) corresponds proportionally to the results of Bastos-Neto et al. (2012), who found 19 morphotypes of bacteria isolated from cassava peel. The result of the logarithmic conversion of CFU reported by these authors was lower (7.4) than the value found in this study (12.8). This fact confirms the microbiological abundance of amylases contained in the sludge, with the potential for biotechnological production of amylase enzymes (ALBUQUERQUE et al., 2010).

For the cellulolytic strains, the results in Table 1 show only two bacterial isolates, but the absolute CFU values were higher (16 log CFU/g sludge). It is worth mentioning that the morphological differentiation of the bacteria was hampered by the dark color of this culture medium and the fact that most bacterial colonies were translucent. There are reports on the isolation of 39 morphotypes of bacteria, of which nine tested positive for degradation of forest litter cellulose (MANTILLA; PINEDA, 2013).

For keratinolytic bacteria, there were 15 isolates and 13.5 log CFU/g sludge (Table 2). These values of the poultry slaughterhouse sludge are considered high for this category. Lucas et al. (2003) found 33 keratinolytic bacterial isolates directly from chicken feathers.

The number of isolates and CFU values were highest for ligninolytic bacteria (Table 2). Few studies have addressed the isolation of lignin-decomposing bacteria. Investigating the biodiversity of bacteria in lignocellulolytic litter of the Atlantic Forest, Martinez (2009) reported 51 morphotypes of bacteria and 8 log CFU/g compost; the same author stated that of the total bacteria, only 20% had the ability to degrade lignin.

Of the bacteria studied, the proteolytic ones had the lowest CFU values (Figure 1), despite the high ability to colonize and persist in different types of environments. Values of 6.5 log CFU/mL were reported in raw milk cooled to 2 °C, indicating a high concentration of this type of bacteria (ARCURI et al., 2008). Along this line of research, 2.3 log CFU/mL were reported in another study on milk cooled to 4 °C (PINTO et al., 2006), reaffirming the ability of proteolytic bacteria to persist under different environmental conditions.

Morphological characterization of bacterial isolates

The 52 bacterial isolates obtained from poultry slaughterhouse sludge were analyzed based on their cell shape and Gram staining, and on the criteria proposed by Perin (2002). The results (Tables 3 and 4, respectively) show high morphological diversity of the bacterial colonies isolated from the sludge from the wastewater treatment plant of the poultry slaughterhouse.

Table 3. Cell characterization of bacteria isolated from sludge from a wastewater treatment plant of a poultry slaughterhouse.

Isolates	Cell shape	Gram stain
Amylolytic	50 % rod 50 % coccoid	100 % positive
Cellulolytic	un.	un.
Ligninolytic	10 % rod 90 % coccoid	90 % positive 10 % negative
Proteolytic	30 % rod 70 % coccoid	90 % positive 10 % negative
Keratinolytic	30 % rod 70 % coccoid	90 % positive 10 % negative

un. : unidentified.

Table 4. Morphological characterization of the colonies of 52 bacterial isolates from the sludge of the wastewater treatment station of a poultry slaughterhouse.

Isolates	Size	Shape	Top	Edge	Surface	Color	Mucoid
Amylolytic	60% ≥ 1 mm 40% < 1 mm	90% round 10% irregular	80% flat 20% lense	70% straight 20% undulated 10% indented	90% smooth 10% rough	100% white	-
Cellulolytic	un.	100% round	un.	100% straight	un.	100% translucent	-
Ligninolytic	100% ≥ 1mm	71% round 29% irregular	64% flat 26% lense	64% straight 21% indented 15% lobed	100% smooth	79% white 21% translucent	-
Proteolytic	89% ≥ 1 mm 11% < 1 mm	67% round 33% irregular	56% convex 44% lense	80% straight 20% undulated	90% smooth 10% rough	70% white 20% cream 10% red	+
Keratinolytic	67% ≥ 1 mm 33%< 1 mm	80% round 20% irregular	60% flat 26% lense 14% convex	93% straight 7% indented	100% smooth	73% white 27% translucent	-

un. : unidentified.
-: absent;
+: present.

Although the bacterial strains had a wide diversity of colony colors, shapes, and sizes, they generally followed a trend, for example, about 80% had a colony size greater than 1 mm, a straight edge, coccoid cell shape, and were gram-positive. A high incidence of gram-positive bacteria was also reported by Faria et al. (2006), in the sludge of sewage treatment plants of Franca and Barueri (77% and 61% of a total of 77 and 143 isolates, respectively). These authors concluded that the most common gram-positive bacteria in the sludge belonged to the genus *Bacillu*s.

This study showed that sludge from wastewater treatment plants of poultry slaughterhouses contains microorganisms that interact and coexist, with specific functions and/or with the potential to degrade various organic compounds or substances. This high diversity allows investigations at several research fronts, with a view to identify biotechnological processes applicable in different fields. One potential area is minimizing the environmental impact of waste in urban and agricultural settings. The by-products from agricultural residues, sewage sludge, and household

waste can be diverted to nutrient cycling processes by the action of microbial organic-matter degraders, thus reducing environmental impacts.

Conclusions

The sludge from wastewater treatment plants of poultry slaughterhouses is a rich source of organo-mineral constituents, and of functional and morphologically diverse bacteria with the potential to degrade organic matter.

The sludge contains, in order of increasing number of types of bacterial isolates: cellulolytic, ligninolytic, amylolytic, keratinolytic, and proteolytic bacteria.

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