Indian Journal of Animal Sciences **90** (4): 530–534, April 2020/Article

Standardisation and categorization of indigenous microorganisms (IMOs) for inoculated deep litter piggery in India

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Received: 25 February 2019; Accepted: 26 August 2019

ABSTRACT

The present experiment was conducted on standardisation and categorization of Indigenous Microorganisms (IMOs) in India for its future application as inoculum in inoculated deep litter housing of pigs. The cultivation of IMOs was accomplished in four steps, which involved use of half cooked rice, sugar sources, rice bran and soil at 1st, 2nd, 3rd and 4th steps, respectively. The cultivated IMOs at the end of each step from 1st to 4th were named accordingly as IMO-1, IMO-2, IMO-3 and IMO-4. The cultivation of IMOs was done in three groups based on the major sources of energy at 2nd step as control (brown sugar), treatment 1 (Jaggery) and treatment 2 (Molasses). The IMO-1 was obtained after 7 days which was confirmed based on the appearance of white coloured fungal mycelium in all the groups. The IMO-4 was considered as the final product of cultivation process which was confirmed by the presence of fungal mycelium interwoven in the soil. IMO-4 stage was further categorised in different microbial groups based on laboratory examination and only two categories of microbes were witnessed namely bacteria and fungi, none of yeast were found in those inoculums. Out of four types of bacterial colonies, IMB-3 confirmed the presence of *Paenibacillus amylolyticus* and IMB-4 as *Enterococcus casseliflavus*. The standardisation of IMOs as inoculum for deep litter housing was performed first time in India.

Keywords: Bacteria, Fungi, Indigenous microorganisms, Inoculated deep litter housing

Indigenous microorganisms (IMOs) refer to a group of locally existing beneficial microbes that constitute important component of world biodiversity (Sadi *et al*. 2006). It is a group of beneficial bacteria, filamentous fungi and yeast which lives together in harmony with the rest of nature and mainly collected from non-cultivated soil. The concept of IMOs has been used basically for natural farming which takes advantage of locally available living microbes to produce fertile soils and higher output without use of herbicides or pesticides. Natural farming with IMOs has been reported to have amazing improvements in soil structure and plant health (Kumar and Gopal 2015). The benefits of using IMOs is that it lowers the cost of production (up to 60%) and produces more desirable crops which are stronger, healthier and more nutritious with zero waste emission. The IMOs have wide range of application such as biodegradation, bioleaching, biocomposting, nitrogen fixation, improving soil fertility and ecofriendly animal husbandry. It has been proved to be beneficial to the farmers to develop sustainable agriculture and crop production (Cho and Koyama 1997). The same principles of IMOs cultivation can also be used in piggery where traditional deep litter may be inoculated to make the sties odour-free and convert the organic waste into better quality compost (Kumar and Gopal 2015). The cultivation and application of IMOs for inoculated deep litter housing has not been attempted in India. Therefore, the present study was conducted to cultivate and standardise Indigenous Microorganisms (IMOs) using different substrates for its future application in inoculated deep litter system (IDLS) of pigs.

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MATERIALS AND METHODS

Selection of site: Cultivation of Indigenous Microorganisms (IMOs) was conducted at Swine Production Farm (SPF) and their standardisation was done at Veterinary Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh (India). The nearby undisturbed area of the farm, surrounded by trees, was selected for cultivation of IMOs.

Materials used for IMOs cultivation: The IMOs in the present study were cultivated by three protocols (groups) and each protocol consisted of four steps (1st, 2nd, 3rd and 4th). The materials used from 1st to 4th step included a wooden box of size 12″ × 12″ × 4″ (volume of 576 cubic inches), half cooked rice, sources of energy (brown sugar/ Jaggery/molasses), rice bran, distilled water, native soil, wire mess, rubber band, white paper etc. The IMOs cultivated at 1st, 2nd, 3rd and 4th steps were named as IMO-1, IMO-2, IMO-3 and IMO-4, respectively based on the

end product of the cultivation. The IMOs were cultivated in three groups based on the readily available source of energy and carbon as skeleton for microbial growth. The step wise protocols used for cultivation of IMOs are as follows.

Cultivation of IMOs in control (*C*): The cultivation of IMOs in control was done in following steps.

1st step (Cultivation of IMO-1): This step involved the cooking of rice, where, a quantity of 2.5 kg raw rice was half cooked and placed in a wooden box of size of 12″ × 12″ × 4″ cubic inches. The box was filled with the half cooked rice up to 2/3rd part and labelled properly. The remaining 1/3rd part of the box was kept empty to facilitate the aerobic environment to the microbes and thereafter the box was covered with a sheet of white (tissue) paper. A wire mess of equal size to the wooden box was placed over and thereafter a thin plastic sheet was also covered to protect the materials from rodents or stray animals in view of avoiding any damage or interference to the contents. The box containing the half cooked rice was buried 5 cm deep in the soil under the trees for 7 days. The top surface of the box was again covered with bamboo leaves collected from the nearby area to maintain optimum moisture for microbial growth. All possible measures were taken to protect the materials from direct sunlight and rainwater while placing it under the native soil.

2nd step (*Cultivation of IMO-2*): It involved the cultivation of IMO-2 which was prepared by mixing IMO-1 (0.5 kg) with brown sugar (0.5 kg) in the ratio of 1: 1. The mixture of IMO-1 with brown sugar (IMO-2) was placed in a cool environment for next 7 days. The enrichment of IMO-1 with energy source further helped in the growth of microbes and fermentation of the substrate.

3rd step (*Cultivation of IMO-3*): In this step, 5 g of IMO-2 was mixed with 500 ml of distilled water until the colour of the solution turned brownish. The completely mixed solution was sprinkled over 4 kg of rice bran to make it semi solid consistency. The materials were covered with rice straw to allow the growth of microorganisms for next 5 days to get IMO-3.

4th step (*Cultivation of IMO-4*): In the last step, 4 kg of IMO-3 was mixed with 4 kg of soil in the ratio of 1: 1. This mixture was allowed to propagate for next 7 days.

Cultivation of IMOs from Treatment 1 (*T1*): The procedure for cultivation of IMOs from T-1 was similar as mentioned in control except at 2nd step, where Jaggery was taken as a readily available source of energy to microbes in place of brown sugar.

Cultivation of IMOs from Treatment 2 (*T2*): The procedure for cultivation of IMOs from T2 was similar as mentioned in control except at 2ndstep, where molasses was taken as a readily available source of energy.

Isolation and broad categorization of IMOs in laboratory: The final products of cultivated IMOs at SPF were further analyzed and broadly categorized in different microbes on the basis of cultural characteristics on different growth media. The laboratory analysis of IMOs was done by standard protocols.

Collection of IMOs samples: The final products of cultivation, i.e. IMO-4 from different groups (from C, T1 and T2) were collected in the sterile glass vials and further processed in laboratory.

Processing of samples containing IMOs: 1 g of IMO-4 (from each group) was collected and thoroughly mixed with 9 ml of Phosphate Buffer Saline (PBS) having pH 7 and molarity 0.1 (0.1 M). All IMO-4 were kept in shaker incubator (Pelican) for 3 h at 200 revolutions per minute (rpm) and the supernatant was collected. After that, 10 fold serial dilutions were made. From each dilution (10^{-1} to 10^{-5}), 100 µl of IMO-4 were plated by spread plate technique.

Different culture media and staining procedures used: The different culture media used were nutrient agar, Sabouraud dextrose agar, Actinomycetes isolation agar, Rose Bengal chloremphenicol agar and staining procedure used were Lactophenol cotton blue staining and Gram's staining. Identification of bacterial colonies was done by VITEK-2 system using standard cards.

RESULTS AND DISCUSSION

Cultivation of indigenous microorganisms (*IMOs*): The IMOs were cultivated successfully from three groups and total duration for cultivation of IMOs (IMO-4) was 26 days. The present study on cultivation of IMOs was carried out during the months of winter, where the duration for cultivation of IMO-1 at 1st step was 7 days. The present study was in agreement with findings of Bakar and Ibrahim (2013) where they prepared IMOs by similar technology for composting. The environmental conditions of the present study were almost similar to the earlier reports on cultivated IMOs, however in contrast to our findings, Hoonpark and Du Ponte (2008) and Anyanwu *et al*. (2013) reported 5 days to complete the 1st step. The duration for cultivation may vary from place to place and depending upon the climatic conditions of the experimental site. The duration for cultivation of IMO-2, IMO-3 and IMO-4 were similar to previous reports (Hoonpark and Du Ponte 2008, Bakar and Ibrahim 2013).

Physical evaluation of IMOs: The cultivated IMOs at various stages were evaluated based on their physical texture and colour. The colour of IMO-1 appeared white due to presence of fungal mycelium in all groups (Fig.1). The physical evaluation at IMO-1 is very crucial as any deviation from the white coloured moulds over the substrate is undesirable for further cultivation of IMOs (Hoonpark and Du Ponte, 2008, Anyanwu *et al*. 2013, Bakar and Ibrahim, 2013). At the end of 2nd step, the IMO-2 was physically evaluated in control, T1 and T2 groups. The IMO-2 in control, T1 had semi-moist but not wet consistency due to less water contents in brown sugar and Jaggery, respectively. However, the consistency of IMOs in T2 was semi-moist in appearance. The colour of cultivated IMO-2 in control, T1 and T2 was dark brown, light brown and light black which might have imposed due to colour of added ingredient like brown sugar, Jaggery

and molasses at this step. The colour of IMO-3 appeared as white patches developed over the rice bran in all the groups. The development of white patches in IMO-3 seems to be due to proliferation of diverse microbes upon enrichment of nutrient.

The IMO-4 stage was the final product of the cultivation process. The colour of IMO-4 was light brown, irrespective of the groups. The fungal mycelum developed on IMO-4 were not prominent as compared with IMO-1 stage; however, the fungal mycelium developed over the substrates were observed as white threads interwoven within the soil. The texture of IMO-4 was similar to granular soil in all the groups. The colour and texture developed at IMO-4 in the present study were positively correlated with previous reports on IMOs cultivation (Hoonpark and Du Ponte 2008, Anyanwu *et al*. 2013). Based on the development of colour mainly the presence of white coloured fungal mycelium, the IMO-1 and IMO-4 stage was considered as positive indicator for successful cultivation of IMOs. The finally cultivated IMOs (IMO-4) were further analysed for counts of bacterial and fungal colonies.

Isolation, identification and characterization of IMOs

Culture media and growth of IMOs: The cultivated IMO-4 was cultured in different growth medium such as nutrient agar (NA) for bacterial colonies and Sabouraud dextrose agar (SDA), Rose Bengal Chloramphenicol agar (RBCA) for fungal colonies and Actinomycetes isolation agar (AIA) for actinomycetes. The two broad categories of microbes were observed, i.e. bacteria and fungi. The NA plates were incubated for growth at 37°C for 24 h whereas the SDA and RBCA plates incubated at 30–37°C for 5 days. The AIA plates could not establish the significant growth of actinomycetes from all groups. The bacterial growth observed in present study was similar as reported by Olufunmi (2013) and Olukunle (2013) whereas fungal growth was similar as observed by Olufunmi (2013). Potato dextrose agar has been used to obtain fungal growth in few studies (Anyanwu *et al*. 2013, Olukunle, 2013). Anyanwu *et al*. (2013) also found the similar results by using Potato dextrose agar (PDA). Bakar and Ibrahim (2013) also reported the absence of yeast in the IMO culture during their investigation, only bacteria and actinomycetes were reported.

Cultural characteristics of bacterial colonies: Four different types of bacterial colonies were observed on nutrient agar plates which were named as IMB-1, IMB-2, IMB-3 and IMB-4. The detailed cultural characteristics of various kinds of bacterial colonies in different groups are given in Table 1. In T1 group, all four types of bacterial colonies were present, however in control only three types of colonies (IMB-1, IMB-2 and IMB-3) and T2 group only two types of colonies (IMB-1 and IMB-2) were present. The diversity of bacterial colonies was higher in T1 group followed by control and T2. The energy source like brown sugar and Jaggery could give better bacterial diversity than molasses; most of the earlier findings were based on brown sugar (Anyanwu *et al*. 2013). The cultural characteristics of microorganisms are important for proper identification of microbes and indicate the adaptation for biological functions oriented towards survival and coping mechanisms of microbes when exposed to varying and different environment (Young 2006). In a similar kind of study, Bakar and Ibrahim (2013) reported broad categories of mesopihilic and thermophilic bacteria.

Total bacterial counts: The bacterial colonies were counted by standard plate count method. The counts of bacterial colonies were represented as number of colony forming units (CFU) per gram. The counts of IMB-1 was highest T1 (15×10^3) followed by control (14×10^3) and T2 (10×10^3). The counts of IMB-2 was higher in control (15×10^3) followed by T1 (07×10^3) and T2 (5×10^3). The IMB-3 was present only in C (3×10^3) and T1 (10×10^3). The IMB-4 was exclusively found in T1 group and the total number of colony counts was 3×10^3. The CFU/g was highest in T1 (35×10^3) followed by control (32×10^3) and T2 (15×10^3). The result indicates that jaggery, which was used as source of energy in T1, promoted sustained growth and multiplication of bacteria. The jaggery gave better results than the brown sugar followed by molasses. The molasses being of poor quality, energy source might not have given similar results as compared with brown sugar and jaggery control and T1, respectively. The CFU in the present study may be correlated with findings of Olukunle (2013) however in which bacterial isolates were analysed in crude oil polluted sites using IMOs based traditional techniques. In a similar kind of study, Anyanwu *et al*. (2013) reported higher concentration of bacteria in bamboo forest using IMOs; however the present study was mainly targeted with nearby pig farm.

Cultural characteristics of fungal colonies: Four different types of fungal colonies were observed on different media used for fungal growth. The observed fungal colonies on SDA were named as IMF-1 and IMF-2 whereas on

Table 1. Cultural characteristics of bacterial colonies grown on nutrient agar (NA) media in different groups (C, T1 and T2)

Bacterial colony	Colour	Colony shape	Size	Texture	Elevation	Appearance	Optical properties
IMB-1	White to cream	Circular	Small (2–3 mm)	Smooth, pasty consistency	Flat	Shiny	Opaque
IMB-2	Cream	Circular	Large (5–6 mm)	Smooth	Flat	Dull	Opaque
IMB-3	Yellow	Circular	Large (4–5 mm)	Smooth	Flat	Shiny	Opaque
IMB-4	Cream	Circular	Small (About 1 mm)	Smooth	Flat	Dull	Opaque

Table 2. Cultural characteristics of fungal colonies grown on SDA and RBCA media in control, T1 and T2

Fungal colony	Colour	Colony shape	Size	Texture	Elevation	Appearance	Optical properties
IMF-1 (SDA)	Off-white at periphery and light khaki at centre	Circular, spreading type	>40 mm	Cotton type	Elevated	No soluble pigments	None
IMF-2 (SDA)	White, no pigmentation	Circular	<10 mm	Cotton type	Elevated	No soluble pigments	None
IMF-3 (RBCA)	Yellowish with white periphery	Circular, spreading type	1–1.7 mm	Powdery	Elevated	No soluble pigments	None
IMF-4 (RBCA)	Off white, brownish appearance with black centre	Circular	0.5 mm	Powdery	Elevated	No soluble pigments	None

RBCA were named as IMF-3 and IMF-4. The detailed cultural characteristics of various kinds of fungal colonies in different groups have been presented in Table 2. All four types of fungal colonies were present in control, T1 and T2. The cultural characteristics of fungi in present study were similar to the findings of Anyanwu *et al*. (2013).

Total fungal counts: The fungal colonies observed on different growth media were counted by standard plate count method. The counts of fungal colonies (per gram) for IMF-1 was higher in control (14×10^3) followed by T2 (5×10^3) and T1 (2×10^3). The CFU/g for IMF-2 was highest in T2 (25×10^3) followed by control (21×10^3) than T1 (17×10^3). The IMF-3 was higher in control followed by T1 and T2. The IMF-4 was highest in T2 followed by T1 and control. The total number of fungal colonies were highest in control (77×10^3) followed by T2 (63×10^3) and T1 (47×10^3). The results in the present study indicates that fungal growth was better in control using brown sugar as a source of energy to the microbes. The present study suggests that fungal growth could also be achieved on molasses despite of inferior energy source.

The development and number of fungal colonies were intermediate in T1 group where jaggery was used. The CFU counts for fungal colonies in the present study was similar to the findings of Anyanwu *et al*. (2013) who studied the concentration of fungal colonies in IMOs near the bamboo forest. Olukunle (2013) reported higher counts of CFU for fungal colonies in crude oil polluted soils using the IMOs.

Total microbial plate counts: Total microbial plate counts both bacterial and fungal colonies were highest in control group (10.4×10^4) followed T1 (8.2×10^4) and T2 (7.8×10^4). The results indicate that growth of fungal colonies was higher in control than other groups. The present findings suggest that soil from the swine production farm, where the IMOs samples were collected was rich in indigenous microorganisms. The growth and multiplication of microorganisms in the soil while cultivating IMOs depend on various physical and climatic factors such as source, temperature, pH, incubation period, nutrient content in the soil, moisture content, etc (Sait *et al*. 2002). Soil with high moisture content and favourable environment conditions are closely associated with high population of indigenous microorganism especially bacteria (Nakasaki *et al*. 2005).

Gram's staining of bacterial colonies: From Gram's staining four types of isolates were identified, out of which IMB-1, IMB-2 and IMB-3 isolates were Gram positive bacilli whereas IMB-4 was Gram positive cocci.

Lactophenol Cotton Blue (*LCB*) *staining of fungal colonies:* From LCB staining, four types of fungal isolates were identified. The microscopic examination revealed that IMF-1 colonies (Fig. 2A) were morphologically similar to

Fig. 1. White coloured fungal hyphae developed at IMO-1 after 7 days.

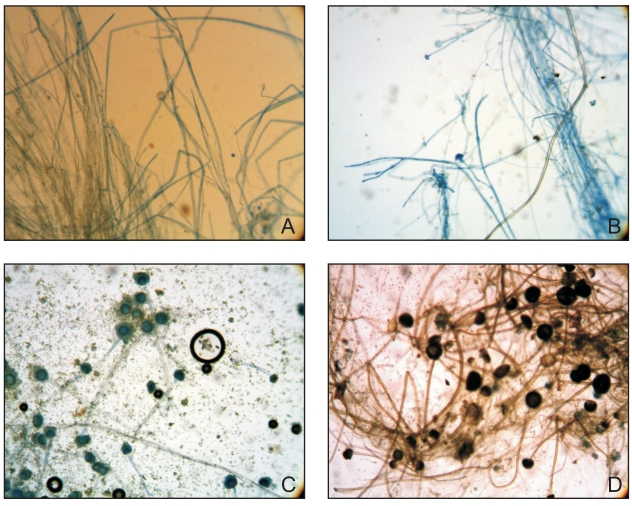

Fig. 2. Morphological feature of fungal colonies. A. IMF-1, B. IMF-2, C. IMF-3, D. IMF-4.

Mucor spp. The IMF-2 colonies (Fig. 2b) had similar morphological structure with *Cunninghamella* spp., whereas IMF-3 and IMF-4 had structural similarity with *Aspergillus* spp. and *Rhizopus* spp., respectively (Figs 2C, 2D). The findings on various isolates of fungal colonies in the present study are in agreement with previous reports where similar group of fungi were isolated having role in biodegradation (Olufunmi, 2013). The presence of these fungal species and their role in biodegradation of organic waste is in agreement with Gaur *et al*. (1982).

Confirmation of bacterial isolates by VITEK-2 system: The bacterial colonies of IMB-3 were confirmed as *Paenibacillus amylolyticus* and IMB-4 as *Enterococcus casseliflavus* using VITEK-2 system. The other colonies like IMB-1 and IMB-2 were of mixed cultures and thus the specific bacteria could not be confirmed. The *Paenibacillus amylolyticus* has role in promoting the crop growth directly via biological nitrogen fixation, phosphate solubilisation etc (Grady *et al*. 2016). The *Enterococcus casseliflavus* is considered a potential probiotic which could have beneficial effect on growth performance, gut health and disease resistance of animals (Safari *et al*. 2016).

From the present study, it is concluded that cultivation of Indigenous Microorganisms (IMOs) can successfully be done during the winter which takes a total of 26 days. The IMOs were broadly categorized in to two groups, i.e. bacteria and fungi having four different types of isolates in each category. This is the first report on cultivation of IMOs to be used for inoculated deep litter system of piggery in India. The IMOs can be inoculated in deep litter materials of pig production system for improving the health, hygiene and comfort of pigs through reduction of bad smell, harmful gases and flies in traditional sties.

ACKNOWLEDGEMENT

The authors would like to thank Director, Indian Veterinary Research Institute, Izatnagar for providing all the necessary infrastructure facilities while carrying out this experiment.

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