Development of Selective Growth Media for Isolation and Enumeration of Biodegraders From Soil

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Abstract

Cellulose and lignin are the main components of crop residues that must be decomposed by heterotrophic microbes either on the surface (zero-tillage) or in the soil (conventional tillage) to release plant nutrients and sustain humus formation. This study was conducted to develop and evaluate improved agar media for the selective enumeration and isolation of a wide range of cellulolytic and ligninolytic microbes from field samples. An existing cellulose-Congo Red agar was modified by replacing the cellulose powder with carboxymethylcellulose (CMC) and by doubling the concentration of soil extract. The new medium (CMCCRA) for cellulolytic bacteria was evaluated, in comparison with the traditional minimal salts-CMC agar (MSCMCA), by spreadplating of dilutions from samples of three, surface-placed crop residues (wheat, pea and flax) and the underlying soil, collected in spring 1996 from a field study at Melfort. Cellulolytic activity was easy to detect on CMCCRA plates due to the distinct zones of clearing around all active colonies, that are clearly visible against a deep red background without having to flood the agar with a liquid reagent as is necessary when using the MSCMC agar. Thus, counts of cellulose degrading bacteria on crop residues and in soil were consistently higher on CMCCRA and also less variable than on MSCMCA. By adding streptomycin we made the CMCCR agar more selective for fungal growth and used it to reliably enumerate cellulolytic fungi, which - as a group - accounted for about 30% of total number of viable fungi in the surface soil.

We also explored the feasibility of using a polymeric dye agar (with Poly R-478) as selective medium for the primary detection of ligninolytic organisms and their subsequent isolation from enrichment cultures.

INTRODUCTION

Over 70 x 10^9 Mg of carbon are fixed annually by terrestrial plants (Paul and Clark, 1996) and a large portion of that phytomass is in the form of cellulose. Thus cellulose is the

main food resource available to heterotrophic microorganisms and the capability of the cellulose degrading community is a useful index of overall decomposition processes (Moore et al., 1979). Changes in degrader populations can, in turn, be related to changes in soil organic matter content, soil health and productivity (Hendricks et al., 1995).

To enumerate cellulose degraders techniques for liquid and solid culture have been developed but most are cumbersome or are not sufficiently sensitive. Several solid culture methods do not allow for direct isolation of organisms from distinct colonies due to the need to flood the agar surface with reagents that effect visualization of cellulose degradation. An earlier study, at this laboratory, on the microbial ecology of decomposing crop residues from a field at Melfort, SK emphasized the need for a more precise and reliable method of enumeration. A minimal salts - carboxymethylcellulose agar (MSCMCA) medium was used in the original analysis. It was found to be cumbersome, required long incubation and a positive reaction (i.e. a zone of clearing around the microbial colony) was often very difficult to discern. A second medium, as described by Hendricks et al. (1995) was also evaluated but was found being too soft to be useful.

Flax is a desirable crop in crop rotations as it breaks disease cycles and has also been referred to as a "clean-up crop" due its ability to reduce weed infestations (Flax Council of Canada, 1996). Flax residue, however, creates problems at seeding of the subsequent crop due to bunching up of straw and clogging of drills especially in zero-till systems. Flax fibres contain lignin or lignocellulose complexes which are very strong and resistant to biodegradation. Fall burning of flax straw is still a common method for getting rid of these persistent residues. However, straw burning pollutes the atmosphere and results in loss of soil protection against erosion, reduced microbial populations (>50% for bacteria) and losses in soil organic matter and nutrients (Biederbeck et al., 1980). If an inoculant could be developed for use after harvest to enhance the breakdown of flax residue (i.e. reduce the tensile strength of the fibres), it would render this crop more suitable for zero-till systems. Fungi with ligninolytic activity have been shown to degrade polymeric dyes which have similar structures to lignins (Glenn and Gold, 1983; Cookson, 1995). Consequently, we examined a possible method for fast and easy detection of ligninolytic organisms, to be used as a prerequisite for monitoring subsequent enrichment (in amended habitats) and eventual isolation of potentially fibre- (or ligno-cellulose-) degrading microbial communities.

The twofold objectives of our studies were to:

1. Develop an agar medium for enumeration and isolation of cellulolytic microorganisms (bacteria and fungi) that allowed easy and precise detection of cellulolytic activity.

2. Develop an agar medium for isolation of potentially ligninolytic microorganisms as the initial bioassay to obtain mixed cultures capable of reducing the tensile strength of flax straw.

MATERIALS AND METHODS

Enumeration of general microflora and cellulose degraders

Five media were prepared and used in our study: soil extract agar (SEA), MSCMCA and carboxymethylcellulose-Congo Red (CMCCRA) with cycloheximide to assess bacterial enumerations and rose bengal-streptomycin agar (RBSA) and CMCCRA with streptomycin to assess fungal enumerations. Soil extract for SEA was prepared from surface soil at the Melfort field site. The MSCMCA was prepared as described by Hankin et al. (1974). The CMCCRA was modified from Hendricks et al. (1995). Earlier attempts to increase the agar strength of the Hendricks medium by addition of more Noble agar resulted in loss of the colour change reaction. Subsequently, we found that using 2 g of high viscosity carboxymethylcellulose (CMC) instead of 1.88 g of acid washed, cellulose powder, as specified in the original recipe, resulted in increased agar strength without any loss in the reaction. The new medium (CMCCRA) consisted of 2.00 g CMC, 0.50 g K_2HPO_4, 0.25 g $MgSO_4$, 0.20 g Congo Red, 5.00 g Noble agar, 2.00 g gelatin, 200 ml soil extract (instead of 100 ml), and 800 ml of distilled water (instead of tap water) for each litre of medium. Since CMC takes a long time to go into solution, we used 200 ml of distilled water to dissolve it while stirring over gentle heat. This solubilization should be done one day prior to making the medium and be placed in a fridge overnight to ensure all of the CMC goes into solution. The remaining ingredients are added while stirring and the medium is tested to ensure it is at pH 7.0. Then the medium is autoclaved for 17 minutes at 121 “C, cooled to 60°C, filter-sterilized cycloheximide (100 μg/ml final concentration) is added for bacterial enumeration, while stirring before dispensing. For fungal enumeration, filter-sterilized streptomycin (100 μg/ml) was added to cooled CMCCRA before dispensing. The final medium should have a deep red colour.

Soil (O-5 cm) and crop residues collected in May 1996 from the Melfort residue decomposition-landscape study (Moulin et al., 1996) were used in this study. For our evaluation of selective media, the colony counts were averaged across all three types of residues (viz. wheat, pea and flax) from the surface-placed bags of the field study. Microbial counts of viable bacteria and fungi were done according to methods described by Biederbeck et a1.(1995) except that the MSCMCA plates were incubated for 21-28 days before the counting of colonies. First all the bacterial colonies on the MSCMCA plates were counted then each plate was flooded with hexadecyltrimethylammonium bromide (HAB) reagent (5 ml/plate) for 30 minutes before the now visible cellulolytic colonies were

counted. Colonies with zones of clearing around them (distinct halo) were counted as being cellulolytic. Colonies on CMCCRA medium were counted in the same manner except that these plates were examined after only 5-10 days of incubation. The ability of this medium to reliably detect cellulolysis was tested using Gliocladium roseum, a known cellulose degrading fungus, as a positive reference culture.

The enumerations were statistically evaluated using analysis of variance (JMP software, SAS Institute Inc., Cary, NC, USA).

Detection Medium for detection of potential flax degraders (Poly R Medium)

This medium was modified from that of Cookson (1995). The polymeric dye, Poly B-41 1, that Cookson used is no longer commercially available so another polymeric dye was substituted. We used Poly R-478, which has a poly(vinylamine) sulfonate backbone plus anthrapyridone chromophore yielding a polymeric structure similar to lignin [Sigma, Oakville, ON]. The modified medium which consisted 15 g malt extract, 10 g agar, 0.2 g Poly R-478 dye and 1000 ml distilled water per litre. The medium was autoclaved at 121°C for 20 minutes, cooled and dispensed into Petri dishes. The resulting medium is reddish-purple in colour. To identify a positive reaction, ***Phanaerochaete chrysosporium*** (ATCC 24725, American Type Culture Collection, Maryland, USA) was used as a reference culture. The inoculated med m was incubated for 5 days at 2 1 °C in the dark.

RESULTS

Evaluation of cellulose degrader media

The growth and enumeration of bacteria and fungi recovered from soil and residue on several different media were examined. Total numbers of bacteria recovered from soil on the solid media decreased significantly (p=O.O5) in the following order: SEA > CMCCRA > MSCMCA at a ratio of 7.7 : 1.8 : 1 (Figure 1). The enumeration of bacteria from the residues followed the same order as above decreasing at a 4.0 : 1.6 : 1 ratio, but numbers of bacteria on CMCCRA and MSCMCA did not differ significantly. As expected, the decomposing crop residues supported an abundance of microbes. Thus, our bacterial counts from the residue samples were found to be about tenfold greater, on all media, than those in the samples of the underlying soil.

The CMCCRA produced a distinct reaction to cellulolysis with a clear zone appearing around each active colony against a deep red background in the agar. As shown in Figure 1, the number of bacteria observed to have cellulolytic activity was higher on CMCCRA than on MSCMCA plates for both soil and residue samples but the difference was most

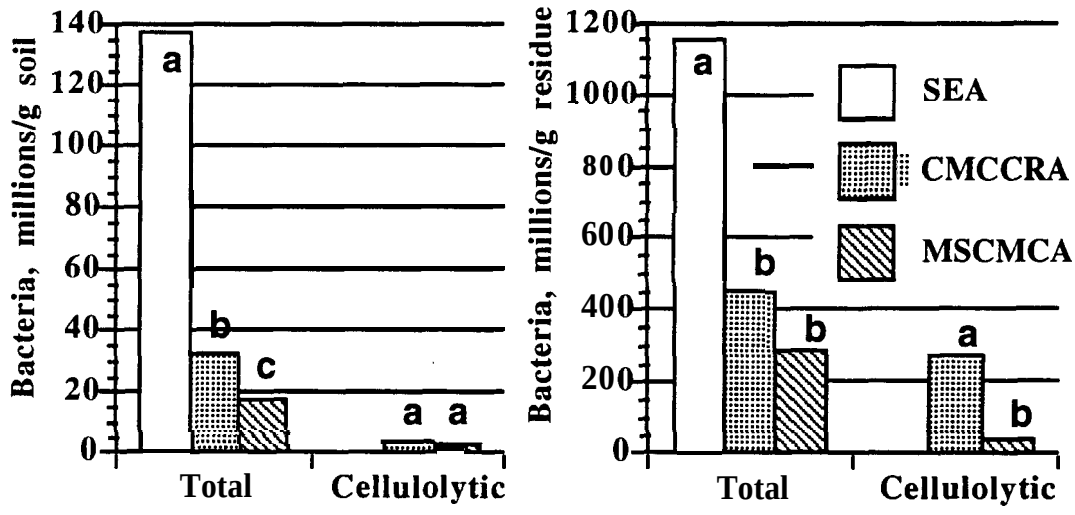

Figure 1. Counts of total and cellulolytic bacteria in A) soil and B) on crop residues sampled at the Melfort site in May, 1996, (bars for total and cellulolytic counts with different letters are significantly different at p=0.05).

pronounced with residues where the new medium produced a sevenfold greater count of cellulose degraders..

In the enumeration of total fungi we found that CMCCRA facilitated the growth of significantly more fungi from soil than did RBSA (Figure 2). About 30 % of all colonies on CMCCRA were found to have cellulolytic activity as indicated by the appearance of distinct clearing around active colonies on a red background.

Evaluation of notential flax degrader medium (Poly R)

The ability of Poly R to identify ligninolytic, potential flax residue degraders was examined. It was found that Poly R did allow for a very distinctive colour change within the medium which is attributed to degradation of the polymeric dye component. The colour of the medium prior to inoculation with ***Phunaerochaete chrysosporium*** was reddish-purple. Upon growth of the lignin degrading fungus there was a distinct change in colour

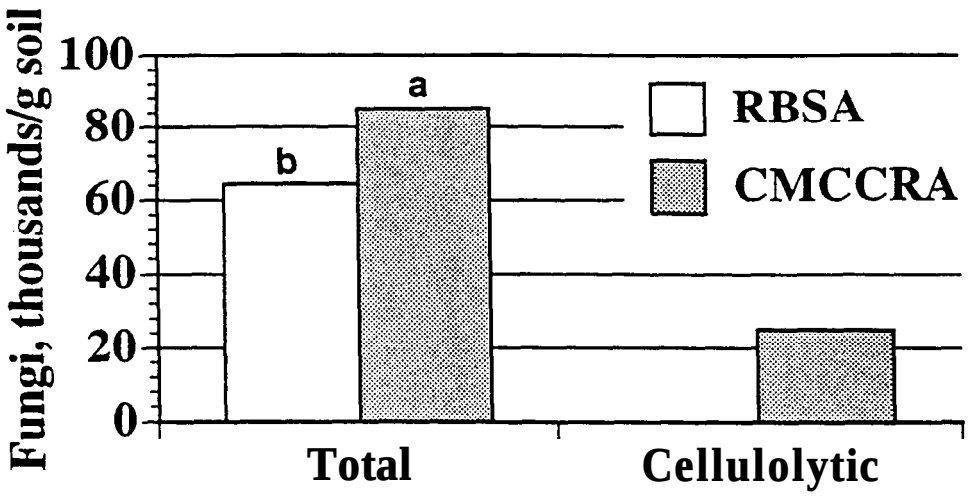

Figure 2. Counts of total and cellulolytic fungi in soil sampled at the Melfort site in May, 1996. (Bars for total fungal counts with different letters are significantly different at p=0.05.)

to a pale yellow. This colour change was not found when other non-ligninolytic, fungal colonies were grown on the same medium. In some preliminary trials, when dilutions of soils from several flax fields were spread on this medium. we observed very low levels of organisms that were able to cause the colour change.

DISCUSSION

The results from this study show that CMCCRA is a much better choice of medium for the selective enumeration of cellulolytic microorganisms compared to MSCMCA. Recovery of total and cellulolytic bacteria was greater with CMCCRA regardless of source. Therefore. CMCCRA is less restrictive than MSCMCA resulting in a more complete representation of the cellulolytic segment of bacterial communities. However, the total number of bacteria counted on CMCCRA was much lower than that found on SEA but this is consistent with the findings of Hendricks et al. (1995).

The addition of streptomycin to make the medium selective for fungi allows for enumeration of the cellulolytic members of that large group of microorganisms. Fungi are known to play a dominant role in cellulose degradation of crop residues on the surface and in the soil. Since MSCMCA could only be used for bacterial enumeration, this medium ignored much of the cellulolytic biota. We were surprised to find that CMCCRA grew

significantly more total fungi than did RBSA which has traditionally been used for general enumeration of fungi (Martin, 1950). In addition to the above advantages, CMCCRA allows for direct isolation of any cellulolytic colony upon being identified as such. With MSCMCA this was impossible due to the need for flooding of the agar surface with the HAB solution to precipitate carbohydrates and visualize zones of clearing with the resultant risk of cross-contamination.

The use of Poly-R medium as a primary indicator of ligninolytic capability shows promise. The very distinct zone of colour change in the medium, found with the ***P. chrysosporium*** inoculated test plates, shows that the reaction is fast and easy to identify. Furthermore, the very low numbers of positive microorganisms, found on dilution plates from fields (even those with flax in the regular rotation), tend to confirm that this may be a good indicator of ligninolytic activity. Possible improvements to the Poly-R medium and use of other selective media are currently being pursued at Swift Current.

Use of both media for isolations is especially important with the growing interest in using agricultural wastes as sources of animal feed, human food and soil-building compost (Isikhuemhen and Zadrazil, 1996). Building a collection of microorganisms or communities of microorganisms with wide-ranging biodegradative capabilities is, therefore, important for future use in research and for potential commercial exploitation.

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