Cassava starch-agar blend as alternative gelling agent for mycological culture media

C.K. Kwoseh; M. Asomani-Darko and K. Adubofour
Department of Crop & Soil Sciences, KN University of Science & Technology, Kumasi, Ghana
#Corresponding author’s email: ckwoseh@hotmail.com

ABSTRACT
Cassava starch has been used successfully in plant tissue culture media, and its potential in culturing fungi was evaluated for cost reduction since agar is expensive. Starch was extracted from blended-peeled cassava var. Dokudwoade paste and sun-dried for 48 hours. The cassava starch alone and its blend with different amounts of agar were used to prepare culture media, and medium with 5g agar/250ml was used as the standard. Each amount of agar and/or cassava starch was added separately to 250ml potato broth prepared from scratch and then autoclaved. Media were dispensed into Petri plates and left over night under ambient conditions and observed for media characteristics. Plugs of 5mm diameter of Aspergillus niger and Fusarium oxysporum placed at the centre of each Petri plate were used to assess growth and sporulation. It was observed that media with higher concentration of cassava starch had difficulty in dispensing. Clarity of the media was generally good. The mycelial growth and sporulation of A. niger on all media were reasonably good. However, growth was profuse on the cassava starch-blend media and this was probably due to additional form of carbon in starch. There was no significant difference (P > 0.05) between colony diameters and sporulation. The aerial growth of F. oxysporum was generally characterised by loose fluffy mycelia. Colony diameter and number of spores of F. oxysporium were significantly (P < 0.05) different. The different growth and sporulation characteristics of A. niger and F. oxysporum may be due to their different nutritional requirements. In general, all the media in the study supported growth and sporulation of the test fungi. The use of cassava starch-agar blend in mycological culture media preparation is therefore recommended after more studies to validate the present results.

Key words: Aspergillus niger, Fusarium oxysporum, sporulation, growth, colony diameter.

INTRODUCTION
The ability to examine and study the characteristics of microorganisms, depend mainly on being able to culture them in the laboratory (Atlas, 1995). Liquid media are difficult to prepare and culture on, identification of microorganisms is difficult and have very high contamination problems. However, solid media makes easy identification of pathological specimen and also reduce contamination (Prescott et al., 2002).

Synthetic media are desirable in bioassay work but most fungi do best on media from natural ingredients (Ritchie, 2002). Most solid media contain an extract of a natural source of carbohydrate and other nutrients to which variable amounts of agar are added to solidify the medium on or in which the pathogen can grow and be observed (Agrios, 2005). According to Ritchie (2002), fungi usually prefer a slightly acid reaction of pH 6 – 6.5 whereas bacteria prefer a neutral pH of about 7. Also, the optimum temperature for growth on artificial media is between 25-30°C.

Desirable qualities of a solidifying agent for media include solidity, transparency, and the ability to form a reversible colloid. Also, the medium must be firm enough to allow
the carrying out of common techniques such as streaking out cultures and plating. In addition, the gelling agent should be relatively inexpensive and easily obtained (Adubofour, 2006; Apiron and Watson, 1976).

According to Mbanaso et al. (2001), cassava starch could be a cheaper alternative to agar in developing countries needing to import agar where cassava is abundant. Kasanadze (2000) and Gerbre and Santhyanarayana (2001) have confirmed the gelling ability of cassava starch and considered it as a potential cheap substitute for agar. According to Mbanaso et al. (2001), solidity of medium increased with increase in starch concentration. Tonukari (2004) reported that a typical composition of the cassava root is moisture (70%), starch (24%), fiber (2%), protein (1%) and other substances including minerals (3%). Kasanadze (2000) found that the quality of the gel was improved by mixing cassava flour with some agar (80g cassava flour + 3.5g agar/litre water).

Agar is not easy to metabolize and has good gel firmness, elasticity, clarity and stability (UNDP/FAO, 1990). However, cassava starch has been reported to have poor paste stability during prolonged cooking (Abraham, 1993). Kasanadze (2000) proved that cassava flour has gelling ability but its ability to support plant tissue or microbial cultures was not evaluated. Solidification of agar within any growth media is reduced as the pH decreases (Rainy and Oren, 2006; Lupano and Gonzalez, 1999). The pH of cassava flour (crude starch) as a gelling agent may drop in pH and it often occurs after autoclaving the media (Kasanadze, 2000). Thus a drop in pH reduces gel stability (Lupano and Gonzalez, 1999).

The competition for agar is very high and its good properties and importance in many industries makes it very expensive. In 2008, the price of 500g of agar on the Ghanaian market ranged between 140 and 160 Ghana cedis (US$1 = Ghc 1). Another cause for concern is the difficulty in the growing of the red sea weeds, Gelidium which are the raw materials for manufacturing agar (UNDP/FAO, 1990). Japan which is the world’s largest producer of agar (UNDP/FAO, 1990) has about two-thirds of agar markers still relying on the natural winter weather to produce agar. Therefore, seasonal changes may cause inconsistencies in importation, and subsequent unavailability of agar on the markets and laboratories. Following these, a more readily available substitute for agar is desirable.

The aim of this study was to find a readily available and cheaper local gelling agent or agar blend for solid culture media preparation. The objectives of this study were to evaluate the gelling ability of cassava-starch blend and, fungal growth and sporulation on media gelled with cassava-starch blend.

MATERIALS AND METHODS

The study was conducted in the Plant Pathology laboratory, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana in 2008.

Preparation of potato broth and potato dextrose agar (PDA)

Potatoes obtained from the KNUST mini market were peeled, washed, sliced into pieces and a 100g sample was put into a beaker and topped with 100ml of water. This was boiled for 30 minutes till it was tender. The potato broth was obtained by straining with a cheese cloth into a conical flask and topped with distilled water to 1000ml. Five gram of agar and 5g of glucose were weighed and put in a 500ml conical flask and then 250ml of the potato
broth added to obtain the PDA which served as the standard.

**Preparation of cassava starch**

The starch used was extracted from cassava variety Dokudwoade obtained from the KNUST Experimental Farm. The tubers were peeled, washed and grated. A blender was used to blend 1kg of the grated cassava into paste. The paste was strained into a clean plastic bucket using a cheese cloth and the solution obtained topped with three litres of sterile distilled water. The starch solution was left in the laboratory under ambient conditions for 24h, after which the supernatant was poured off to obtain a clean starch paste. The starch was broken into pieces and sun-dried for 48h and crushed into powder to enhance solubility. The dried starch powder was used for the study.

**Media preparation**

Two levels of the starch (20g or 40g) were amended with either (1g or 2g agar), and 250ml of potato broth. Potato dextrose agar (PDA) served as the standard. The following were the treatments;

- a. 20g cassava starch + 1g agar
- b. 20g cassava starch + 2g agar
- c. 40g cassava starch + 1g agar
- d. 40g cassava starch + 2g agar
- e. 40g cassava starch alone PDA (standard)

The pH values of all media were measured before and after autoclaving using a pH/mV/TEMP meter (Suntex SP-701 model).

**Pre-heating of media, autoclaving and dispensing onto the Petri plates**

The media preparations were pre-heated separately on a gas cooker and continuously stirred with a stirring rod for six minutes till it became thickened. This was to ensure a homogenous mixture, increase its elasticity and to eliminate the tendencies of lumping during autoclaving. The different media preparations in conical flasks were stoppered with cotton wool and autoclaved at 121°C and 1bar pressure for 20 minutes. The sterilized media were dispensed separately into 9cm diameter sterilized Petri dishes and left for 24h to solidify in a sterilized transfer chamber. Each Petri dish contained about 25ml of the medium.

**Culturing of test fungi on media**

Three-day-old Aspergillus niger (van Tieghem) and Fusarium oxysporum (Schltdl.) cultured on PDA were used as test fungi. A flame-sterilised 5mm diameter cork borer was used to cut fungal plugs from the periphery of the fungal cultures and these transferred to plates using a flame-sterilised needle. Insulating tape was used to seal the plates to prevent contamination and the plates inverted to minimise condensation in a sterilized transfer chamber at 25-26°C in day light and darkness for seven days.

**Experimental design**

A Completely randomized design (CRD) with three replicate plates per treatment was used for the study. The data were analyzed using GENSTAT version 11 statistical package (2008) and least significant differences at 5% were used to compare mean differences. The experiment was repeated two times.

**Data collection**

**Clarity of mycological culture media in Petri plates**

This was based on how well the test fungi could be seen by unaided eye on the various media. The cultures were observed from below the plates. The clarity of the growth of fungi on media was compared to the standard PDA. The scoring system below was used for clarity.

1= Organisms absolutely seen (Excellent)
2= Organism seen (Very Clear)
3= Organism partially seen (Clear)
Organism cannot be seen (Opaque)

Measurement of colony diameter of test fungi

The colony diameter in cm was determined at seven days of growth by marking out two diagonals perpendicular to each other across the base of the 9cm diameter Petri plate, meeting at the point where the fungal plugs were placed. A ruler was used to measure the radial growth of the fungi in each plate along the diagonals and the mean per plate calculated.

Estimation of sporulation of test fungi

A 5mm-radius cork borer was used to make three discs from each plate on the seventh day of growth. Each of the discs was taken about 1cm from the centre of the plate. Three samples from each culture plate were then bulked in a 20ml McCartney bottle and put through serial dilution using sterile distilled water. The dilution was done five times with 10^{-5} as the final serial dilution. A microscope was used for counting the spores on a haemocytometer slide, and mean spore counts (x) per unit disc were square root transformed (\sqrt{x+1}) and used for analysis.

Determination of aerial mycelia growth of test fungi

The measurement of the aerial growth was based on the appearance of the vegetative growth of the test fungi. This was done using visual observation by a panel ranking of ten persons based on the scoring system below:

<table>
<thead>
<tr>
<th>Scoring system of aerial growth of test fungi</th>
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</thead>
<tbody>
<tr>
<td>Aerial growth characteristics of test fungi</td>
</tr>
<tr>
<td>Poor growth (Mycelium sparse)</td>
</tr>
<tr>
<td>Good growth (Mycelium moderately extensive)</td>
</tr>
<tr>
<td>Very good growth (Mycelium extensive)</td>
</tr>
<tr>
<td>Profuse growth (Mycelium very extensive)</td>
</tr>
</tbody>
</table>

RESULTS

Viscosity and pouring ability of mycological culture media

The PDA standard had light viscosity and as such was easy to dispense into plates. The 20g cassava starch +2g agar and 20g cassava starch +1g agar media were also light in viscosity and were easy to dispense just like the case with the standard. However, the 40g cassava starch +2g agar medium was quite difficult to dispense and the 40g starch +1g agar medium was much more difficult to dispense because the molten media were just smearing the lining of the flask.

Solidification (solidity) of mycological culture media

The consistency of the various media in plates was assessed visually. It was observed that the 20g cassava starch +2g agar medium solidified very well and there was no significant difference (P > 0.05) in comparison with the standard (PDA). The 40g cassava starch +2g agar medium also solidified well compared with the PDA. Unlike the PDA, the surface of the 40g cassava starch +2g agar medium was dominated by whitish patches of starch. The 20g cassava starch +1g agar medium although solidified, was broth in nature. The 40g cassava starch alone plates became watery with bad odour four days after inoculation so they were discarded.

<table>
<thead>
<tr>
<th>Table 1. pH of mycological culture media before and after autoclaving</th>
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</thead>
<tbody>
<tr>
<td>Media (Amount in 250ml potato solution)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5g agar (PDA)</td>
</tr>
<tr>
<td>20g cassava starch +1g agar</td>
</tr>
<tr>
<td>20g cassava starch +2g agar</td>
</tr>
<tr>
<td>40g cassava starch +1g agar</td>
</tr>
<tr>
<td>40g cassava starch +2g agar</td>
</tr>
</tbody>
</table>
Generally, the pH of all the media dropped after autoclaving (Table 1). In general, all the media were very clear for the growth patterns of the organisms to be seen and measured (Table 2).

<table>
<thead>
<tr>
<th>Media (Amount in 250ml potato solution)</th>
<th>Score (Scale 1-4)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5g agar (PDA)</td>
<td>1</td>
</tr>
<tr>
<td>20g cassava starch +1g agar</td>
<td>1</td>
</tr>
<tr>
<td>20g cassava starch +2g agar</td>
<td>2</td>
</tr>
<tr>
<td>40g cassava starch +1g agar</td>
<td>2</td>
</tr>
<tr>
<td>40g cassava starch +2g agar</td>
<td>2</td>
</tr>
</tbody>
</table>

*1 (excellent), 2 (very clear), 3 (clear), 4 (opaque)

### Aerial mycelia growth and colony diameter of *A. niger* on mycological culture media

The mycelia in *A. niger* aggregated into tight pellets that grew as spherical balls and branching was restricted to the surface pellets. The media generally supported very good aerial growth of *A. niger* although some media exhibited profuse growth (Table 3). The colony diameter of *A. niger* on the seventh day ranged between 85mm on the PDA and 79.5mm on 40g cassava starch+2g agar media (Table 4). The colony diameters of *A. niger* on all media were not significantly different (**P > 0.05**) (Table 4).

### Aerial mycelia growth and colony diameter of *F. oxysporum* on mycological culture media

The growth pattern in this fungus was characterized by loose and fluffy mycelia. Aerial mycelia growth was generally very good on all test media (Table 3). The colony diameter of *F. oxysporum* ranged between 57.8mm on 20g cassava starch +1g agar medium and 71.0 mm on the PDA (Table 4). There were significant differences (**P < 0.05**) between the colony diameters on the PDA and 20g cassava starch +1g agar medium as well as 20g cassava starch +2g agar and 40g cassava starch +2g agar media. However, there was no significant difference (**P > 0.05**) between the 40g cassava starch + 1g agar medium and PDA (Table 4).

### Sporulation of *A. niger* on mycological culture media

The transformed mean number of spores of *A. niger* per disc was between 7.9×10³ on the PDA and 6.7×10³ on 20g cassava starch+2g agar medium (Table 5). There was significant difference (**P < 0.05**) between the number of spores on PDA and 20g cassava starch+2g agar medium. However, there were no significant differences (**P > 0.05**) between the rest of the media and PDA (Table 5).

### Sporulation of *F. oxysporum* on mycological culture media

The transformed mean number of spores recorded was between 5.3×10³ on the 40g cassava starch + 1g agar and 4.1×10³ recorded on the 20g cassava starch +2g agar medium (Table 5). Although, there was no significant difference (**P=0.05**) between the PDA and 40g cassava starch + 1g agar and 40g cassava starch + 2g agar media, these
treatments had higher sporulation rate than the PDA. However, there was significant difference (P=0.05) between 40g cassava starch +1g agar and 20g cassava starch +2g agar media (Table 5).

Table 4. Colony diameter of A. niger and F. oxysporum on mycological culture media at seven days after plating

<table>
<thead>
<tr>
<th>Media</th>
<th>Mean colony diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. niger</td>
</tr>
<tr>
<td>5g agar (PDA)</td>
<td>85.0</td>
</tr>
<tr>
<td>20g cassava starch+1g agar</td>
<td>82.3</td>
</tr>
<tr>
<td>20g cassava starch+2g agar</td>
<td>81.5</td>
</tr>
<tr>
<td>40g cassava starch+1g agar</td>
<td>83.2</td>
</tr>
<tr>
<td>40g cassava starch+2g agar</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Table 5. Mean number of A. niger and F. oxysporum spores per disc at seven days after planting on mycological culture media

<table>
<thead>
<tr>
<th>Media</th>
<th>Mean no. of spores <em>(x+1)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. niger</td>
</tr>
<tr>
<td>5g agar (PDA)</td>
<td>7.9</td>
</tr>
<tr>
<td>20g cassava starch +1g agar</td>
<td>7.3</td>
</tr>
<tr>
<td>20g cassava starch +2g agar</td>
<td>6.7</td>
</tr>
<tr>
<td>40g cassava starch +1g agar</td>
<td>7.6</td>
</tr>
<tr>
<td>40g cassava starch +2g agar</td>
<td>7.6</td>
</tr>
</tbody>
</table>

DISCUSSION

According to UNDP/FAO (1990), the viscosity of an agar broth at constant temperature and concentration is a direct function of the average molecular weight. In relation to this reference, the difficulty in dispensing of media with the higher concentration of starch was due to increase in the viscosity of the media.

The surface of the higher cassava starch + agar medium was dominated by whitish patches of starch and this might have been due to the higher concentration of starch in the medium. The 20g cassava starch+1g agar medium did not solidify. UNDP/FAO (1990) reported that the gel strength of agar varies with the concentration used. The 40g cassava starch, without agar did not solidify. The plates became watery in appearance after the fourth day and were not used for further studies. Kanasadze (2000) observed that media gelled with cassava flour lost their gel and the solid particles of flour deposited at the bottom of the tubes. Mbanaso et al. (2001) made a similar observation with cassava starch gelled medium after seven days. Lupano and Gonzalez (1999) reported that this is probably due to drop in pH which often occurs after autoclaving the media. The pH of all the media dropped after autoclaving. This confirms the findings of Lupano and Gonzalez (1999) that a drop in pH reduces gel stability.

The radial growth of A. niger was very rapid which might have been due to nutritional factors, as the cassava starch could be serving as an additional carbon source for the fungus. The colony diameters of A. niger on all media were not significantly different (P > 0.05) and this agrees with the observation by Adubofour (2006).

The media generally supported very good aerial growth of A. niger although some media exhibited profuse growth. According to Onwueme (1982), starch provides 35% carbohydrate and 1% mineral matter and therefore the starch might have been an additional carbon source to the medium promoting good growth. Tonukari (2004) also reported that a typical composition of the cassava root is moisture (70%), starch
The slow growth of F. oxysporum might be explained by the fact that the nutritional requirement of this fungus is different from what was provided in the form of cassava starch. There was also no significant difference (P > 0.05) between the 40g cassava starch + 1g agar medium and PDA with respect to the colony diameter of F. oxysporum but the other treatments were significantly different (P < 0.05). These observations may be due to the nature of the substrate, pH, and accumulation of waste products which affected the linear branching of mycelia (Griffin, 1981). According to Agrios (2005), pH affects fungal cell growth and development of F. oxysporum. There was sporulation of F. oxysporum on all the media including the standard (PDA) tested. However, the 40g starch supported more sporulation of the fungus probably because starch may not be rich in nutrients or too rich in nutrients required for F. oxysporum to encourage sporulation at the expense of vegetative growth. According to Carlile et al. (2001) and Griffin (1981), it is usually very difficult to relate experimental observations on the control of fruiting to natural conditions that stimulate sporulation, considering the lack of information on the chemical and physical status of natural microenvironment inhabited by fungi. Ritchie (2002) reported that some fungi require high nutrients to sporulate at the expense of vegetative growth. According to Wanatabe (2002), most deuteromycetous fungi may sporulate on rich agar media.

CONCLUSIONS
Cassava starch showed some level of gelling ability. The cassava starch alone cannot be used for culture media unless blended with some amount of agar. Culture media with higher concentrations of cassava starch without agar could not gel. Preheating and continuous stirring of media before autoclaving as well as pouring the media hot must be done for easy pouring of plates. All the media in the study supported the growth and reproduction of F. oxysporum and A. niger.

The cassava starch-agar blends used in the study can be used for mycological culture media preparation to reduce the cost of media after further tests.

RECOMMENDATIONS
- Other local gelling sources such as yam, maize and sweet potato starch should be evaluated.
- Further tests on the cassava starch-agar blends for mycological culture media preparation should be done

REFERENCES