



Anaerobic digestion of sugar beet – fate of plant pathogens and gas potential

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ABSTRACT

Sweden and Europe aims at increasing the use of renewable energy. Biogas represents one way to reach this goal. Biogas, produced from organic waste or crop materials, can be used for production of heat and electricity and as fuel for vehicles. The biogas process is also advantageous as it mediate the recirculation of nutrient from waste products to arable fields. This can be achieved by spreading the nutrient rich bio manure, which is left after digestion, on arable fields. Organic material, used as substrate in a biogas process, can contain different contaminating organisms such as different fungi. Some fungi are plant pathogens and if these survive the biogas process and is spread on arable land they might infect the new crop, thus leading to reduced yields and an increased need for fungicides. If storage pathogens are spread they may survive on organic debris on the ground and damage the harvested crop during storage. Therefore it is important to evaluate potential risks when materials infected with plant pathogenic fungi are used as substrate in a biogas process. Furthermore, if fungi are killed, the biogas process offers an alternative way of using crops with not good enough quality for food or feed production. Presently, it is however at unclear what levels of gas production that can be reached with such “low quality” materials.

The aim of this study was to investigate fate of plant pathogens during mesophilic anaerobic digestion and also to investigate gas production potential of infected and uninfected sugar beet, both fresh beet roots and those stored for one year. Survival studies were performed for three different sugar beet field pathogens, *Aphanomyces cochlioides*, *Pythium ultimum* and *Rhizoctonia solani*, causing emergence diseases, and for two different storage pathogens *Fusarium culmorum* and *Botrytis cinerea*. The gas production potential was determined in a batch test system started with inoculum from two different large scale biogas plants.

The measurement of gas production potential showed that both uninfected fresh and stored sugar beets produced more methane (per g added Volatile Solid) than beet material infected by the different fungal pathogens. Survival studies performed with spores of *Fusarium culmorum* and *Botrytis cinerea* demonstrated a very short survival time, less than 2.5 hours. For two sugar beet pathogens, *Aphanomyces cochlioides* and *Pythium ultimum*, it was not possible to obtain the most resistant survival structure, the oospores, and the survival test was therefore performed with only mycelia and/or oogonia. Both these structures survived for a very short time.

In order to predict the fate of these fungi in a biogas process, more studies are needed. However, even though it was not possible to test all fungal structures of interest, the results so far suggest that it is unlikely that fungi would pose a great problem in bio manure. Before the material is digested and used as bio manure it passes several steps, sanitation, anaerobic digestion, post-digestion, aerobic storage, with varied environments. Therefore, it seems unlikely that a fungus can adapt and survive through all of those steps. Conclusively, a biogas production process could be a good way to dispose of contaminated organic material. However, it is important to consider the lower methane yield when planning the biogas plant.

Keywords: biogas, bio residue, sugar beet, plant pathogen, *Aphanomyces cochlioides*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium culmorum*, *Botrytis cinerea*.

SAMMANFATTNING

Både Sverige och Europa har som mål att öka andelen förnyelsebar energi och ett sätt att uppnå detta är genom en ökad biogasproduktion. Biogas kan produceras genom rötning av gödsel, energigrödor eller organiskt avfall. Den kan användas till uppvärmning, elektricitet och som fordonsbränsle. Kvar efter rötningen finns en näringsrik rötrest. Genom att använda denna restprodukt som gödningsmedel till jordbruksgrödor är det möjligt att få cirkulation av näringsämnen. Det organiska materialet som används i biogasproduktionen kan innehålla olika föroreningar, som till exempel svampar. En del svampar är växtskadegörare och dessa kan, om de överlever rötningen och sprids med rötresten, infektera en ny gröda. Som en följd kan detta leda till skördesänkningar och ett ökat behov av bekämpningsmedel. Om lagringssvampar sprids med rötresterna kan dessa kanske överleva i marken och, om de följer med grödan vid skörd, också orsaka skador under lagring. På grund av detta är det viktigt att utvärdera vilka risker som finns med att använda svampinfekterat material för biogasproduktion. Om svamparna däremot dör kan en biogasprocess vara ett bra sätt att ta hand om organiskt material med dålig kvalitet, som inte kan användas till mat eller foderproduktion. I dagsläget är det dock oklart hur stor gasproduktion ett sådant material ger jämfört med oinfekterat material.

Syftet med detta arbete var att undersöka överlevnaden av svampar under mesofil rötning samt att undersöka gasproduktionspotentialen för oinfekterade och infekterade sockerbetor, både färska betor och sådana som lagrats ett år har testats. Tre olika fältsvampar, *Aphanomyces cochlioides*, *Pythium ultimum* och *Rhizoctonia solani*, som orsakar groddbränna på sockerbetor, och två lagringssvampar, *Fusarium culmorum* och *Botrytis cinerea* undersöktes i överlevnadsstudierna. Gasproduktionspotentialen bestämdes genom satsvisa biogassystem som startades med rötresten från två olika storskaliga biogasreaktorer.

Mer total gas och metan producerades från de oinfekterade betorna (per g tillsatt organiskt material) än de som infekterats med olika svampar, detta gällde både för färska och lagrade betor. Överlevnadsstudierna med sporer från *Fusarium culmorum* och *Botrytis cinerea* visade att de överlever mindre än 2,5 h. För två av skadegörarna på sockerbetor, *Aphanomyces cochlioides* och *Pythium ultimum*, gick det inte att odla överlevnadsstrukturer, oosporer. Därför gjordes överlevnadsstudier med mycel och oogonia. Båda dessa strukturer överlevde emellertid mycket kort tid.

För bedöma potentiell överlevnad av dessa växtpatogener behövs fler studier. Trots att det i studien inte gick att pröva överlevnaden av alla intressanta svampar och deras strukturer indikerar ändå resultaten att de sannolikt inte utgör något större kvalitetsproblem i rötresten. Dessutom består röttningsprocessen oftast av flera olika steg: hygienisering, rötning, efterrötning och aerob lagring innan den används som gödselmedel. I dessa steg är miljöförhållandena väldigt olika och därför är det inte troligt att en svamp klarar alla dessa olika miljöer. Eftersom svampen antagligen inte är ett problem så borde biogasproduktion vara ett bra sätt använda infekterat material. Det är dock viktigt att vid beräkningar och dimensionering av biogasreaktorn och dess ekonomi ta hänsyn till att en lägre metanmängd produceras från detta material.

Nyckelord: biogas, rötrest, sockerbeta, växtpatogener, *Aphanomyces cochlioides*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium culmorum*, *Botrytis cinerea*.

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1 INTRODUCTION

1.1 Background

This Master Thesis was performed within the *MicroDrivE* research program (MicroDrivE.slu.se) at the Department of Microbiology, Swedish University of Agricultural Sciences (SLU). *MicroDrivE* is collaboration between SLU and several sectorial organisations and companies, co-financing the program. The overall goal of the research within this programme is to improve the processes of biogas and ethanol production, including substrate storage and pre-treatment, process efficiency as well as utilization of residues. Hereby, the amount of renewable energy produced in Sweden potentially can be increased and the development of a sustainable society is facilitated.

1.2 Aim

In this master thesis work the biogas process has been in focus, particularly the quality of the digestion residues (bio manure). Biogas plants can operate with different substrates such as manure, food- and slaughter house waste, energy crops etc. Energy crops can, during storage or in the field, become infected by various fungi. Very little research has been performed concerning the fate of such contaminating fungi during digestion of organic material in a biogas process. Fungi surviving a biogas process can represent a risk as they might infect new plants when the biogas digestion residue, i.e. bio manure, is spread on agricultural fields. Furthermore, if fungi are killed, the biogas process could be one way of using crop material with not good enough quality to be used in the food nor feed industry. However, it is presently unclear if plants infected with fungi have the same biogas potential as uninfected plants.

To resolve these questions the aim of this Master Thesis was to

- A) compare the amount of gas produced from sugar beet of good and poor quality
- B) investigate survival of plant and storage pathogens during anaerobic digestion.

1.3 Biogas

The European Commission has decided that in 2020 20 % of the energy used should be from renewable source (Börjesson & Mattiasson, 2008). The goal for the transport sector is that renewable fuels should correspond to a minimum of 10 % of the total fuel used. At the moment the main biofuels used are bioethanol, biodiesel and biogas, i.e. the first generation of bio fuels. It is believed that these will later be replaced by more resource efficient bio fuels from lignocellulose, the so called second generation of fuels. To reach the European Commissions goal it is important to increase the amount of energy produced from crop materials. Energy crops as a substrate for biogas production is energy efficient as it can generate twice as much net energy yield per hectare as can bioethanol or biodiesel (Börjesson & Mattiasson, 2008).

Several major environmental goals can be fulfilled with establishment of biogas processes; a) reduction of the amounts of emitted carbon dioxide by replacement of fossil fuels by biogas in vehicles and for production of electricity and heating (Baky et al, 2006); b) reduced use of mineral fertilizers by utilization of bio manure, thereby also reducing the emissions of climate gases such as N₂O and giving a recirculation of nutrients between rural and urban areas (Baky et al, 2006; Börjesson & Mattiasson, 2008), and; c) decreased methane emissions from

manure storage tanks by instead using the manure as substrate in a closed biogas system (Börjesson & Mattiasson, 2008). Methane is a more potent green house gas than carbon dioxide why manure as substrate for biogas production followed by burning of the produced methane to carbon dioxide gives a reduction of climate gases (Brandt & Gröndahl, 2000). According to calculations the total biogas potential in Sweden is 14-17 TWh, 80 % of this is estimated to come from agricultural substrates such as straw, ley and manure (Nordberg, 2006). Accordingly, the greatest biogas production potential is found in the dominant agricultural areas, such as Skåne, Östergötland and Västergötland.

1.3.1 The biogas process

Biogas is formed during anaerobic digestion of organic materials (Zinder, 1984). The gas is mainly composed of methane and carbon dioxide. The biogas process occurs in natural environments such as wetlands and swamps but is also used in constructed reactors for degradation of different types of organic materials. For constructed processes different process types exist, of which continuous and batch system are of most interest for this work. The continuous process is the most commonly used large scale system in Sweden (Nordberg, 2006). In continuous biogas plants new substrate is added continuously and simultaneously digestion residue is taken out (Hansson & Christensson, 2005). The average time the substrate remains in the biogas reactor is called the hydraulic retention time (HRT). As material continuously is taken out, all organic material will not have time to be completely degraded. Thus, the residue taken out is commonly transferred to a post-digestion treatment tank. Here, undigested materials will have a chance to be converted into biogas, which also is collected. Finally the digestion residue is stored in an ordinary storage compartment (Hansson & Christensson, 2005). In a batch system all substrate is added at one time and remains in the digester until completely degraded. Biogas reactors are most commonly run at mesophilic (35-40 °C) or thermophilic (55-60 °C) temperature. Depending on process type and digesting material the HRT can vary between 10-30 days (Nordberg, 2006). During the biogas process the nitrogen and minerals are conserved in the biogas digestion residue, also called bio manure (Svensson et al, 2005).

There are four main microbial groups involved in the conversion of organic material into biogas (Zinder, 1984). The first group hydrolyses complex organic compounds into different monomers, e.g. sugars, amino acids. The second group, the fermentative bacteria, converts these monomers into acetate, short chain fatty acids, alcohols, carbon dioxide, hydrogen gas etc. The third group performing so called anaerobic oxidation, converts acids and alcohols into acetic acid, hydrogen gas and carbon dioxide. The last microbial group consists mainly of two different groups of methanogens. One group produces methane from acetic acid and contributes to approx. 2/3 of the total methane production in a biogas reactor. The other methanogenic group produces methane from carbon dioxide and hydrogen gas. For the biogas process to function properly, all these microbially mediated reactions must proceed in a synchronized manner. Accumulation of intermediate products can result in a complete process failure.

1.3.2 Substrate

The most common substrate at Swedish biogas plants is presently manure and slaughter house waste (RVF Utveckling, 2005b). Other substrates used are for example municipal household- and restaurant waste, waste from the food industry and byproducts from the bio fuel industry, i.e. glycerol and distiller's waste (RVF Utveckling, 2005b; Börjesson & Mattiasson, 2008). Different crops such as sugar beet, ley, maize, cereals, potatoe and hemp can also be used for

biogas production (Biogassyd homepage, 2008). The crop should optimally have a low level of lignin, a compound not possible to degrade in a biogas process. The carbon/nitrogen quota (C/N quota) of the substrate is also important for the process and should be 15-30 (Hansson & Christensson, 2005). Both high and low levels of nitrogen can inhibit the biogas process. The substrate composition not only has an influence on the process performance but also affect the composition of the produced biogas (Biogassyd homepage, 2008). For example, materials rich in fat or protein give a high level of methane, 70 % and 80 %, respectively, as compared to carbohydrate rich substrates giving only around 50 % methane in the gas. The amount of organic matter added to a biogas process is commonly given as volatile solids (VS) (Biogassyd homepage, 2008). Volatile solids are the dry matter content minus the ash content given in % of total fresh weight. A higher methane yield per kg VS is often achieved during co-digestion of substrates compared to separate digestion of the same materials.

The production of sugar beet requires approximately 12 MWh/ha & year (Nordberg, 2006). When sugar beet are used in a biogas reactor they yield approximately 44 MWh/ha & year. If the tops also are to be included in the biogas processes, the amount of energy would be 53 MWh/ha & year. The quota between added and produced energy is therefore 3.7 and 4.4 respectively. Sugar beet and its tops can give 450 m³ of methane/ton VS (Biogassyd homepage, 2008). This is more than other crops tested, i.e. ley, maize or wheat. To preserve the sugar beet tops and other materials, until use in the anaerobic degradation, they can be ensiled (Svensson et al, 2005).

1.3.3 Using biogas

Biogas can be used as vehicle fuel, for heating and for electricity production (Nordberg, 2006). One m³ methane gas is equal to 1 liter of oil, which is about 10 kWh (Biogassyd homepage, 2008). When biogas is to be used for heating only low methane level (20 %) is required (Nordberg, 2006). When the gas is to be used for production of electricity, the methane level in the gas must be above 40 %, a concentration commonly reached in biogas plants. Different kinds of engines can be used and the efficiency is 30-40 % and the rest of the biogas is converted to heat. When gas is to be used for heat and/or electricity production it has to be cleaned from water and hydrogen sulfide in order to increase the lifetime of the engines used. To be used as vehicle fuel the methane level in biogas has to be above 97 %. To reach this level the gas needs to be cleaned from carbon dioxide, water and hydrogen sulfide.

1.4 Bio manure

1.4.1 Sanitation and certification

Ingoing material to a biogas plant can contain viruses, bacteria, fungi etc (RVF Utveckling, 2005b). To avoid that these possible harmful organism survive the biogas process and ends up in the digestion residue, the material is commonly sanitized by a preceding pasteurization step (Sahlström, 2003). During pasteurization the material is heated to 70 °C for 1 h. Pasteurization is, according to Swedish law, obligate if the material contains animal byproducts, such as manure or slaughter house waste. To ensure a good sanitation the pasteurization is preferably done batch wise. Pasteurization can reduce the amount of pathogens and indicator organisms below detectable level but often it does not reduce the amount of bacterial spores, such as *Clostridium* (Bagge et al, 2005) or fungal spores (Schnürer & Schnürer, 2006). Although pathogens are killed during pasteurization they can reappeared in storage tanks for bio manure at farm level (Bagge et al, 2005). This may be due to two reasons; a) that organisms survived the pasteurization but were reduced to concentrations below detection level, or b) that a

recontamination has taken place. As the vehicles used are difficult to clean between ingoing and outgoing transports, recontamination can occur during the transport by car/truck to farm storage tanks. Of the possible contaminants, only bacteria and fungi has the possibility to survive and grow in the soil environment, if they would be spread with bio manure (RVF Utveckling, 2005b). The decrease of viable bacteria in biogas plants is dependent on temperature, treatment time, pH, volatile fatty acids (VFA), digestion system (batch/continuous), bacterial species, available nutrient and the initial amount of pathogen (Sahlström, 2003). High levels of ammonia have also been shown to be of importance for the killing of pathogens (Ottoson et al., 2008). Temperature is the most important factor for reduction of pathogens, why a more efficient hygienisation is achieved in a thermophilic biogas process as compared to a process operating at mesophilic temperature. High level of VFA with a low pH can also decrease the bacterial number in a biogas process (Fukushi et al., 2003).

The bio manure can be certificated by SPCR 120, SP Swedish National Testing and Research Institute (SPs Certifieringsregler för biogödsel, 2007). When certified, the producers are allowed to sell the bio manure with the lable “Certifierad Återvinning” (Certificated Recycling). To be certified, the bio manure should be produced from clean organic wastes such as manure, silage, household waste etc. It should also contain low levels of different metals, visible contaminants and viable seeds. Furthermore, values for total amount of nitrogen, phosphorous and potassium, amount of organic matter and pH etc. should be given when the bio manure is sold. For certification, also analysis of the number of indicator bacteria (*E. coli* or *Enterococci*, indicating fecal contamination) should be performed as a routine monitoring at the biogas plant. The certification has contributed to acceptance for the use of bio manure by Sigill, Cerealia and Findus (RVF Utveckling, 2005a).

1.4.2 The composition

The composition of bio manure will vary somewhat depending on the material used for the anaerobic digestion (RVF Utveckling, 2005a). Due to these variations it is important to characterize every batch of bio manure and not use values from a few tests. Since most biogas residues have high water content, 93-98 %, large volumes need to be spread to cover the plants nutrient demand (Svensson et al, 2005; RVF Utveckling, 2005a). To avoid compaction, due to application of large volume and weight, the bio manure should contain at least two kg ammonium nitrogen (NH₄-N) per ton and the total nitrogen (Tot-N) content should be three to four kg per ton (Baky et al, 2006). When comparing bio manure from biogas plants with beef and pig slurries (Table 1) it is clear that it has a comparably good nitrogen content with a high degree of NH₄-N, easily available for the plants (RVF Utveckling, 2005a).

Table 1. Average nutrient content in bio manure from a biogas plant and in slurries from beef and pig. The values are given as amount per ton wet weight (RVF Utveckling, 2005a; Kirchmann, 2008).

Manure	DM %	Tot-N kg/ton w.w.	NH₄-N kg/ton w.w.	Phosphorus kg/ton w.w.	Potassium kg/ton w.w.
Bio manure	2-7	4.5	3.0	0.50	2.0
Beef slurry	7-12	3.9	1.8	0.75	4.0
Pig slurry	7-12	5.1	3.3	1.9	3.0

1.4.3 Using bio manure

Most farmers that use bio manure as fertilizing agent are positive concerning its quality and effects (RVF Utveckling, 2005a). They think that it smells less than ordinary manure, give better nitrogen effect than slurry and is easier to spread. Most often the bio manure is spread during spring tillage and incorporated into the soil. It can also be spread in growing crop, which reduces the risk for soil compaction. When spread in growing crop, the farmers normally apply mineral fertilizer when the crop is sown and then bio manure when the crop is 15-30 cm high. There are diverging opinions about the most efficient use of bio manure. According to some it should be used in combination with mineral fertilizer at sowing (RVF Utveckling, 2005a; Svensson et al, 2005) but according to others the yield is higher when bio manure is spread at sowing rather than in a growing crop (Baky et al, 2006). The bio manure is mostly applied with a band application spreader (RVF Utveckling, 2005a). To reduce nitrogen losses the bio manure should optimally be incorporated into the soil (Berg, 2000). When incorporating it within 8 h after spreading, the loss is only about 10 % of the total nitrogen. To decrease ammonia losses during storage, the containers with bio manure should be covered by a roof or straw (RVF Utveckling, 2005a; Berg, 2000).

Bio manure have been tested and compared with mineral fertilizers in several field trials and mostly the yield has been the same for both types of fertilizers (RVF Utveckling, 2005a). Bio manure is most efficient for crops with a short intensive growth and nitrogen uptake, such as barley (Svensson et al, 2005), but it also seems to be a very good fertilizer for sugar beet, potatoes and cereals (RVF Utveckling, 2005a). Some farmers also use bio manure on leys. When used on leys there are restrictions, due to risk of spreading *Salmonella* bacteria, concerning time between spreading until grazing or harvesting (RVF Utveckling, 2005a & 2005b). Due to the low phosphorus content in bio manure it commonly needs to be complemented with a mineral phosphorus fertilizer in order to maintain the phosphorus state in the ground (Svensson et al, 2005).

1.5 The sugar beet crop

Sugar beet, *Beta vulgaris*, is a biennial plant (Cooke & Scott, 1993). During the first year it produces a rosette of leaves and a storage root and carbohydrates produced via photosynthesis are stored as sucrose in the root. During the second year, the sugar beet will flower and produce seeds, if experiencing a vernalisation.

The world supply of sucrose, commonly called sugar, is mainly produced from sugar cane and sugar beet (Cooke & Scott, 1993). Sugar cane is grown in tropical and subtropical regions while sugar beet is better adapted to temperate regions. Growing sugar beet allows many countries to be less dependent on imported sugar. Naturally the sugar content of sugar beets is only 4 % of the fresh weight. However, through breeding the sugar content has been increased to 18-20 %.

1.5.1 Sugar beet in Sweden

In Sweden, sugar production started on a large scale during the last decades of the nineteenth century and the first large sugar factory was founded in 1853 (Cooke & Scott, 1993). Today sugar beets are only grown in the southern parts of Sweden, however it can also be cultivated further north. The reason for cultivation only in the south is simply due to the localisation of the sugar factory. However, the sugar beet yield and development are favoured by a long growing season, typically obtained in the southern parts of Sweden. A sugar beet crop can

produce more than 45 tonnes fresh weight sugar beet roots per hectare, from which about 8 tonnes of sugar can be extracted (Fogelfors, 2001). In UK the tops and crowns from sugar beet constitute around 50 t/ha, corresponding to 5-6 t dry matter (Cooke & Scott, 1993) and are usually ploughed-in at the field (Svensson et al, 2005). However, they can also be feed to animals (Cooke & Scott, 1993) or used in biogas reactors (Hansson & Christensson, 2005).

In Sweden beet roots needs to be stored before delivery to the factory (Cooke & Scott, 1993). The sugar beet campaign, at the only sugar factory left in Örtofta in Skåne, normally runs from September to January and the farmer have a set period when to deliver the sugar beet (Haraldsson, pers mes, 2008). To make it equal for the different farmers and to spread the work at the factory over a longer season, the delivery time for each farmer varies between different years. To maximise the yield, the farmer wants to leave the beet plants in the ground for as long time as possible (Cooke & Scott, 1993). However, the beet needs to be harvested before any serious frost damages them. The sugar beet can withstand a few degrees below zero for a short time (Fogelfors, 2001). However, if it experiences a temperature below -5 °C for a longer period it will be damaged and when it defrosts it cannot longer be used for sugar production. Furthermore, sugar beet that is damaged by frost cannot be stored without a great risk for deterioration caused by microorganisms. During storage, the sugar content of the beet roots is decreased through respiration, healing of wounds from harvesting, enzymatic conversions and growth of storage pathogens (Klotz & Finger, 2004). Respiration accounts for approximately 50-60 % of sugar losses (Cooke & Scott, 1993). About 10 % of the total losses can probably be attributed to storage pathogens, mainly *Botrytis cinerea*, *Phoma betea* and *Penicillium* species (Cooke & Scott, 1993). A lot of sugar is also lost by dehydration, especially of the outer 60 cm of the pile.

1.5.2 Sugar beet pathogens effecting the early stand

Since the monogerm seed were developed the sugar beet crop is seeded to a stand, meaning that no thinning is required or desired (Cooke & Scott, 1993). Unlike cereals and rape seed, sugar beet does not have a great compensational growth, i.e. if one seed does not produce a plant, the neighbouring plants cannot fully compensate by growing more. It is therefore important to establish all seedlings. One important reason for all seeds not producing plants is damping-off diseases, i.e. when young plants are attacked and killed by pathogens as they emerge (Ewaldz, 1987). Damping-off symptoms are caused by both seed carried pathogens, as *Phoma betae*, and soil carried pathogens, as *Aphanomyces cochlioides*, *Pythium spp.* and *Rhizoctonia solani*. In 1987 Ewaldz collected samples from Swedish sugar beet fields and the three most common genera of infecting fungi in these samples were *Aphanomyces* (25 %), *Pythium* (30 %) and *Rhizoctonia* (23 %). It has also been shown that the occurrence of *Aphanomyces* and *Pythium* are lower in high yielding fields than in average fields (Amein, 2006). Damping-off can partly be controlled by including fungicides in the seed coat (Gustafsson, 2007). However, the best way to stop the build up of pathogens is by crop rotation, with at least four years between sugar beet and other susceptible crops. By just looking at the symptoms it is very difficult to distinguish between infections by *Aphanomyces* and *Pythium* (Ewaldz. 1987). Attacks of *Pythium* are usually observed first. Later, when the temperature has reached 15 °C, the attacks of *Aphanomyces* begin. Total attacks of damping-off are highest 1-2 weeks after sowing (Ewaldz, 1987).

1.6 *Aphanomyces cochlioides*

1.6.1 Damage by *Aphanomyces cochlioides*

A. cochlioides generally does not affect seedling emergence of sugar beet plants but more often infects and cause damping-off 1-3 weeks after emergence (Cooke & Scott, 1993; Mukhopadhyay, 1987). At this early stage infection often results in wilting and death of the plant (Mukhopadhyay, 1987). Symptoms caused by the infection are darkening water-soaked areas on the hypocotyls and down on the tap root. The wilted seedling shrivels very quickly and can only be seen as a dark thread. As this scenario is very similar to poor germination it is difficult to estimate the losses caused by *A. cochlioides*. If the seedlings survive, they are stunted and will have reduced growth the rest of the season. Later in the season, the surviving seedlings may develop chronic root rot and wilting. *A. cochlioides* may also cause new infections later in the season when it infects the tap root and causes tip rot (Mukhopadhyay, 1987). The sugar beet will then look as if it has scars and often it has a very thin root (Persson, 2002). The infections of plants are caused by zoospores that are attracted to the beet root by exudates of organic acids and carbohydrates (Ewaldz, 1987). The zoospores can be released from the oospore and one oospore close-by the beet root is enough to produce inoculum for an infection. The initial inoculation often takes place through the hypocotyls at the soil surface. Infection by *A. cochlioides* is favored by damp conditions, as zoospores needs free water to germinate (Mukhopadhyay, 1987), and high temperatures between 17-25 °C (Ewaldz, 1987). Therefore, early sowing is one way to decrease the risk for infection (Mukhopadhyay, 1987). *A. cochlioides* is found in higher frequency in soils with low pH and in organic soils compared to clay soils (Ewaldz, 1987). *A. cochlioides* is considered to be strongly affected by crop rotation and to reduce risks for infection alfalfa and beans should be avoided as pre-crops (Mukhopadhyay, 1987). Several other cultivated crops and weeds are also susceptible to *A. cochlioides* infections (Ewaldz, 1987; Mukhopadhyay, 1987).

1.6.2 Fungal characteristics

A. cochlioides has both a sexual and asexual reproduction (Ewaldz, 1987). The asexual stage constitutes a short time period in the lifecycle; when the infected tissue starts to collapse the fungus shifts to sexual reproduction. During the asexual stage, zoospores are formed inside a sporangium (Mukhopadhyay, 1987). When the primary zoospores emerge from the sporangium they form a spherical structure. After 1-3 h secondary zoospores are formed. These are 13 µm long and have a diameter of 7-8 µm. Initially, the zoospores have two flagellas and therefore they can swim around in search for a suitable place to germinate (Cooke & Scott, 1993). The transformation into sexual stage happens in older rotted tissue. The first step is formation of reproductive organs named oogonia (female structure) and antheridia (male structure) on the mycelia. This will result in a nearly colorless oospore, 16-24 µm in diameter, with a 1.5-3 µm thick wall (Mukhopadhyay, 1987). The oospore is the survival structure of this fungus and can survive for more than one year in the soil (Hovi, 2005). The zoospore may survive for 10 days in the ground (Ewaldz, 1987).

1.7 *Pythium ultimum*

1.7.1 Damage by *Pythium ultimum*

Pythium ultimum is present in nearly all cultivated soils (Whitney & Duffus, 1998) and causes seed rot and pre- and post-emergence damping-off in sugar beet (Mukhopadhyay, 1987). The pre-emergence damping-off is very difficult to spot and is often mistaken for low seed viability (Mukhopadhyay, 1987). In Finland the yield reduction due to *P. ultimum* is on

average 2-3 t/ha (Luterbacher et al, 2005). The symptoms of post-emergence damping-off are slightly darkened water-soaked spots, 1-3 cm below the soil surface (Mukhopadhyay, 1987). The seedlings are either killed quickly or they recover with non affected growth. The fungus mostly causes damping-off at low temperatures, below 15 °C, and at low pH (Ewaldz, 1987). At low temperature and in soils with low pH the sugar beet plants will have reduced growth, thereby being more susceptible to infection by *Pythium*. There seems to be a tendency for greater damage on fine soils as for example clay soil. *Pythium* is also favored by high soil moisture (Ewaldz, 1987; Whitney & Duffus, 1998) and it is considered a crop rotation disease as it has a very broad range of host plants including both cultivated species and weeds (Ewaldz, 1987). Apart from all host plants, *Pythium* is also a natural soil inhabitant and can grow saprophytically in the ground if the water content is high (Ewaldz, 1987; Mukhopadhyay, 1987).

1.7.2 Fungal characteristics

Pythium ultimum is one of at least twelve *Pythium* species that have the capacity to infect sugar beet plants causing damping-off (Amein, 2006). It has both sexual and asexual reproduction (Ewaldz, 1987). During the asexual reproduction a sporangia develop, which germinate either by producing zoospores or directly by germ tube formation. The zoospores are kidney-shaped and have two flagella enabling them to swim towards the sugar beet root. The germination is triggered by root exudates, such as sugars and amino acids. When transforming into sexual reproduction, oogonia and antheridia are formed on the same hypha (Ewaldz, 1987). From the oogonia, a yellowish oospore is developed, 14-19 µm in diameter, with a 1.5-3 µm thick wall (Whitney & Duffus, 1998; Mukhopadhyay, 1987). The oospore cannot germinate until it has experienced a resting period. After that, it can germinate by a germ tube which will form mycelia, which happens in warm weather (~28 °C), or the germ tube can develop into zoospores, which happens in cooler weather (Ewaldz, 1987). The asexual sporangium seems to function both as survival structure and as an infective agent. Unlike the oospore, the sporangia do not need a resting period and the quick germination of the sporangia helps *P. ultimum* reach the substrate before a natural antagonism is developed. The sporangia can survive for 11 months and germinate quickly after addition of nutrients.

1.8 *Rhizoctonia solani*

1.8.1 Damage by *Rhizoctonia solani*

Rhizoctonia solani can cause root- and crown rot, damping-off and foliar blight in sugar beet plants (Cooke & Scott, 1993). The damping-off can be pre-emergence, but usually *R. solani* attacks the plant after it has emerged. The infection often starts below the soil surface and then continues up along the hypocotyls, where a sharp border between infected and healthy parts can be seen (Whitney & Duffus, 1998). Severe attacks will kill the plant but this is not common in Sweden as the fungus is most aggressive in warm climates (21-29 °C), and rarely infects below 15 °C (Hovi, 2005). The risk for damping-off by *R. solani* is greatest with late sowing in warm soils (Cooke & Scott, 1993). Later in the season, this fungus can cause crown rot and dry rot canker on the sugar beet root (Whitney & Duffus, 1998). The fungus can spread, with soil, between and within fields through wind, machines, irrigation water etc (Cooke & Scott, 1993). The fungus can also cause disease in several different crops and due to this it is regarded as a crop sequence fungus (Cooke & Scott, 1993). It has been shown that there is higher risk for attacks when the pre-crop has been beans, legumes, maize and other susceptible crops compared to cereals (Whitney & Duffus, 1998; Ithurrart et al, 2004).

1.8.2 Fungal characteristics

Rhizoctonia solani is a basidiomycete that does not produce spores (Hovi, 2005). The fungus can be recognized by its very characteristic mycelia, with branching at nearly right angles and a septum close to the branching point. When *R. solani* infects a plant, the mycelia first grow over the surface and then compact masses of hyphae, so called infection cushions, are formed (Cooke & Scott, 1993). From the cushions, cell wall-degrading enzymes are released and infection pegs can penetrate the host. It can survive as hyphae or sclerotia on organic debris in soil (Cooke & Scott, 1993). *R. solani* are divided into several different groups and subgroups called anastomos groups (AG), based on the capability for hyphal cells to fuse, known as anastomos (Andersson, 2001). This fusion can only take place between hypha from the same group. The different groups prefer different hosts, i.e. AG 2-2 and AG 4 preferably infects sugar beet and AG 3 have a specificity for potato (Andersson, 2001; Buttner et al, 2002). AG 4 causes damping off in seedlings and AG 2-2 causes root and crown rot (Buttner et al, 2002).

1.9 Botrytis cinerea

1.9.1 Damage by Botrytis cinerea

Botrytis cinerea is mostly a saprophyte, i.e. growing on dead material, but it can also be a weak parasite (Johansson & Andersson, 2003). Throughout most of the world, *B. cinerea* is the most destructive storage pathogen on sugar beet (Cooke & Scott, 1993). It can infect at both high and low temperatures and is therefore a very aggressive storage pathogen (Persson, 2002). It often grows on the surface where the root tip has been broken off. However, as the infection also is influenced by other factors, such as the climate, not all mechanical wounds are attacked. Furthermore, sugar beet roots grown under the right nitrogen conditions are more resistant to *B. cinerea* (Cooke & Scott, 1993). Respiration increases in roots infected with storage pathogens. There can be a 100 % increase in respiration throughout the entire root if 20 % of the root surface is infected. In this respiration, the sugar accumulated in the root is consumed and therefore the sugar content decrease.

B. cinerea can infect and cause losses in more than 200 different plant species around the world (Williamsson et al, 2007). The attacks are often referred to as grey mould because the mycelia is grey and airy (Johansson & Andersson, 2003). Mainly dicotyledons are affected, for example root fruits, vegetables, protein-rich legumes, small fruit and cut flowers (Williamsson et al, 2007). *B. cinerea* can grow for long periods at temperatures just above freezing. In the large international trade fruits and vegetable are stored for long times in cold-rooms therefore *B. cinerea* represent an increasing problem. The fungus is most destructive on mature and senescent tissue but can also attack plants in field. When *B. cinerea* destroy harvested plants, it has often gained entry to the tissues much earlier and remained quiescent for a considerable period of time. It becomes active when the environment becomes favorable as the plants physiology changes during maturation.

1.9.2 Fungal characteristics

Botrytis cinerea is an ascomycete that produces two kinds of conidia (Williamson et al, 2007). The macroconidia contains several nuclei and the microconidia are uninucleate. Often the conidia are the most important propagule (Elad et al, 2004). The fungus can form apothecia but this is not so common in field (Williamson et al, 2007). Apothecia are the structures where the sexual ascospores are formed. For apothecia to form, the fungus has to be exposed to low temperatures (Johansson & Andersson, 2003). On ageing structures, the fungi can form chlamydospores (Elad et al, 2004). These are formed from vegetative mycelia and can

function as short term survival structures. *B. cinerea* can form sclerotia and these, together with mycelia on crop residues, are important survival structures (Johansson & Andersson, 2003). The sclerotia are formed on dying host tissue and can survive desiccation, UV radiation and microbial attacks (Williamson et al, 2007). In early spring, the sclerotium germinates and produces conidia that serve as primary inoculum. The production of conidia is favored by UV-light and it is mostly dispersed by air currents, either individually or in clumps. When *B. cinerea* attacks a plant, it gains entry by secreting cell-wall-degrading enzymes that breach the surface (Williamson et al, 2007). A high germination rate for the conidia is favored by high humidity, i.e. 93-100 % (Johansson & Andersson, 2003). The conidia of *B. cinerea* have been tested for survival at different temperatures (Marquenie et al, 2002). At the lowest temperature tested, 40 °C, the decimal reduction time was 30 minutes.

1.10 *Fusarium culmorum*

1.10.1 Damage by *Fusarium culmorum*

Fusarium culmorum is a soil borne strong saprophyte and only weakly specialized as a pathogen (Leonard & Bushnell, 2003). It is normally not a pathogen on sugar beets but has been found on stored and damaged beets (Persson, 2002). *F. culmorum* has, however, been found to invade draught-affected roots and cause wilting of the plant in the UK (Cooke & Scott, 1993). Sugar beet plants with black necks during harvesting and storage are often infected by *F. culmorum* (Persson, 2002). In storage trials, losses of 18 % of fresh weight have been reported for sugar beets infected by *Fusarium* for storage at +2 °C for 97 days. The weight loss for uninfected sugar beet was 13.1 %. Storage at higher temperature, +12 °C, gave much higher weight losses, 36 % of fresh weight for *Fusarium* infected sugar beet (Persson, 2002).

F. culmorum is present worldwide in the soil, roots and heads of grasses (Leonard & Bushnell, 2003). It is mostly found on crop residue, but sometimes also in the soil or on living tissue. *F. culmorum* are known to produce at least two different mycotoxins (Hörberg, 2001). Mycotoxins can be produced in sugar beet tissue infected by *Fusarium* (Bosch & Mirocha, 1992). This can happen both on rotten tissue in fields and during storage (Bosch & Mirocha, 1992) and the toxins can remain in beet products after processing. The toxins can cause diarrhea, vomiting, weight losses and fertility problems (Hörberg, 2001; Bosch & Mirocha, 1992). *F. culmorum* can also infect several other crop species and weeds (Leonard & Bushnell, 2003; Hörberg, 2001). The most important are maize and cereals.

1.10.2 Fungal characteristics

F. culmorum is an ascomycete but does not, as known so far, have a sexual stage with ascospores (Leonard & Bushnell, 2003). On high carbon substrates, it produces a fast growing red mycelium. *F. culmorum* only produces macroconidia and these are typically banana-shaped and about 35 µm long. The conidium has a mucilaginous sheath, which may improve nutrient uptake. The fungi can produce sporodochia on potatoe dextrose agar. *F. culmorum* can also produce thick walled cells called chlamydospores, which are survival structures during harsh conditions. Different isolates of *F. culmorum* differ in infection aggressiveness (Leonard & Bushnell, 2003).

Growth of *F. culmorum* can start from macroconidia, hyphal fragments and chlamydospores (Hörberg, 2001). The conidia can be spread 90 cm horizontally by rain splashes and longer by wind. The macroconidia germinate fast, 5-6 h, on the plant by forming a germination tube

(Leonard & Bushnell, 2003). The greater number of conidia that are infecting a plant, the shorter the incubation period will be. Later in the infection, *F. culmorum* increase in vigor and there is a change to necrotrophic stages in the colonization of plants. This change is associated with the formation of the mycotoxin DON, which can be spread by water movement in plants and thus can, after about 10 days from infection, be found in parts without visible infection.

2 MATERIALS AND METHODS

2.1 Fungal strains and growth conditions

Botrytis cinerea J613 and *Fusarium culmorum* J618 were obtained from the culture collection at the Department of Microbiology, SLU. They had originally been isolated from sugar beet in storage. *Aphanomyces cochlioides*, *Pythium ultimum* and *Rhizoctonia solani* were obtained from Syngenta Seeds, Landskrona, Sweden. Two fungi previously isolated (Zetterström, 2008) from a residue from a biogas plant in Hohenberg-Krusemark, Germany were also included in the survival study. The biogas plant was operating with mainly energy crops (maize silage) and the presence of these two fungi in the residue indicated good survival through the process. The fungi were in this study identified through sequence analysis of the 18S rRNA genes and showed 98 % genes homology with *Rhizomucor variabilis* and *Mucor circinelloides*, respectively, and these names will be used throughout the study, although the identities need to be further confirmed.

Growth and sporulation media for the different fungi, included in the study, are shown in table 2 and 3. To prevent bacterial growth, chloramphenicol (100 mg/L; Sigma) was added to medium used during plating of digestion residues in survival test (2.3). *B. cinerea*, *F. culmorum*, *R. variabilis* and *M. circinelloides* were incubated at 25 °C and for sporulation the plates were incubated at room temperature under UV-light. *R. solani* was incubated at 25 °C and both *A. cochlioides* and *P. ultimum* were incubated at 20 °C, all three in complete darkness to simulate their natural habitat. Plates with fungi from natural infected stored sugar beet (SSB) were incubated at 25 °C.

Table 2. General growth media used for fungi included in the study.

	Media	Abbreviation	Manufacturer	Reference
<i>Botrytis cinerea</i>	Malt Extract Agar	MEA	Oxoid	-
	Malt Extract Agar w. ox bile	MEA/ox	Oxoid/Merck	Appendix 1
<i>Fusarium culmorum</i>	Malt Extract Agar	MEA	Oxoid	-
	Malt Extract Agar w. ox bile	MEA/ox	Oxoid/Merck	Appendix 1
<i>Aphanomyces cochlioides</i>	Corn Meal Agar	CMA	Difco	-
<i>Pythium ultimum</i>	Corn Meal Agar	CMA	Difco	-
<i>Rhizoctonia solani</i>	Potato Dextrose Agar	PDA	Oxoid	-
<i>Rhizomucor variabilis</i>	Malt Extract Agar w. ox bile	MEA/ox	Oxoid/Merck	Appendix 1
<i>Mucor circinelloides</i>	Malt Extract Agar w. ox bile	MEA/ox	Oxoid/Merck	Appendix 1
Fungi from naturally infected stored sugar beet (SSB)	Malt Extract Agar w. ox bile	MEA/ox	Oxoid/Merck	Appendix 1
	Potato Dextrose Agar	PDA	Oxoid	-

2.2 Extracting spores from fungi

Spores would in general be more likely to survive the biogas process than mycelia and they are thus of greater interest to test in survival trial. Therefore spores from *Botrytis cinerea* and *Fusarium culmorum* growing on OMA were collected in peptone water (deionized water, 0.2 % peptone and 0.01 % Tween 80) and quantified by counting in a Bürkners chamber. Likewise, spores were collected from *Rhizomucor variabilis* and *Mucor circinelloides*, growing on MEA, and the spores were counted.

For *Aphanomyces cochlioides* it would be of most interest to test their survival structure, the oospore, in survival studies. For production and collection of oospores, two different methods were evaluated: 1) OMA with growing *A. cochlioides* was mixed with peptone water in a blender. The mixture was then filtrated through a polyester filter with a pore size of 75 µm. The material collected in the funnel was re-suspended in peptone water and filtered through a filter with pore size of 580 µm. Here everything, i.e. both agar and fungal structures, passed through the filter. Both fractions (from 75 µm and from 580 µm filter) were saved. 2) Mycelia from *A. cochlioides* growing in OMB were rinsed with peptone water four times. After washing, the mycelia were mixed with new peptone water in a blender for 10 min. The mixture was then filtered through a 75 µm filter. The material collected in the funnel was also saved. All material was stored at + 4 °C until use, within 24 h. The solutions, obtained from the different methods, were studied microscopically and the one showing the most interesting structures was later used for survival tests (2.3). Unfortunately, oospores were not seen in any of the fractions. The OMA mixture passing through 580 µm was the one containing most mycelia and oogonia and this fraction was chosen for the survival studies of *A. cochlioides*.

As for *A. cochlioides* the oospores from *Pythium ultimum* is the most interesting structure to test in survival studies. Also here two methods were evaluated for the production and collection of oospores; 1) PDA/S or P₅ARP agar media with growing *P. ultimum* was mixed with peptone water in a blender for 5 min and the obtained solution was then filtrated in a serie through several different polyester filters having 74 µm, 150 µm and 580 µm large pores. The smallest pore size was initially used. The material collected in the funnel, after filtration, was re-suspended in peptone water before repeating the filtration using a larger pore size. Everything, i.e. both fungal structures and agar, passed through the filter with largest pore size. All fractions were saved separately. 2) Mycelia of *P. ultimum* growing in V8 broth were washed four times with peptone water. After washing, the mycelia were mixed with new peptone water for 10 min in a blender. The mixture was then filtered using a 75 µm filter. The material collected in the funnel was also saved. All materials were stored on ice at +4 °C until use within 24 h. As for *A. cochlioides* none of the solutions contained oospores, as determined by microscopy. The agar mixture from P₅ARP, which had passed through the 580 µm filter, contained most mycelia and oogonia and was used in survival studies.

To obtain infectious material of *Rizoctonia solani* the fungi were grown on barley kernels with ox bile. The barley kernels, infected with *R. solani*, were incubated in the dark at 25 °C for about one month. During this time the jar was shaken from time to time in order to spread the fungus in the whole jar. The barley kernels were then dried for 15 days in an aluminum tray placed in a fume hood at room temperature. Finally, the kernels were grounded in a coffee grinder and stored in closed vials at room temperature. The obtained powder was later used in different survival trials. Kernels were also used directly after incubation without drying and grinding.

Table 3. Sporulation media and incubation time.

	Media	Abbreviation	Modification	Reference/ Manufacturer	Structures produced	Incubation time
<i>Botrytis cinerea</i>	Oat Meal Agar	OMA	-	Difco	Conidia Mycelia	10 days
<i>Fusarium culmorum</i>	Oat Meal Agar	OMA	-	Difco	Conidia Mycelia	10 days
<i>Aphanomyces cochlioides</i>	Oat Meal Broth	OMB	Made from oat gruel	Singleton et al, 1992	Mycelia	25 days
<i>Aphanomyces cochlioides</i>	Oat Meal Agar	OMA	-	Difco	Mycelia Oogonia	40 days
<i>Pythium ultimum</i>	V8 juice broth	V8	V8 juice was replaced by Fontana mixed vegetable juice	Khan et al, 1993	Zoospores Mycelia	40 days
<i>Pythium ultimum</i>	Potato Dextrose Agar w. cholesterol	PDA/S	-	Appendix 1	Zoospores Mycelia	25 days
<i>Pythium ultimum</i>	Pimaricin-Ampicillin-Rifampicin-PCNB	P ₅ ARP	-	Singleton et al, 1992	Zoospores Mycelia Oogonia	20 days
<i>Rhizoctonia solani</i>	2 % Pepton-2 % Sucrose-0,5 % Yeast extract	PSY	-	McCoy & Kraft, 1984	Mycelia	20 days
<i>Rhizoctonia solani</i>	Barley kernels w. ox bile	Barley kernels	-	Appendix 1	Mycelia	24 days
<i>Rhizomucor variabilis</i>	Malt Extract Agar w. oxbile	MEA/ox	-	Appendix 1	Conidia Mycelia	30 days
<i>Mucor circinelloides</i>	Malt Extract Agar w. oxbile	MEA/ox	-	Appendix 1	Conidia Mycelia	30 days

2.3 Survival studies in anaerobic batch reactors

Survival of pathogens during anaerobic digestion were investigated in lab-scale batch cultures using residues from two different biogas plants in Norrköping (N) respectively Västerås (V). The plant in Norrköping operates on wheat grain and distillers residue (from an ethanol production facility) and the plant in Västerås operates on organic household waste and grass silage. Survivals of pathogens were investigated by addition of either spore suspension, agar mixture with fungal structures, infected barley kernels or sugar beet to the batch cultures (Table 4).

The amounts of viable fungi at different time after incubation were determined by counting the number of cfu in samples taken out after different incubation times. The first sample was withdrawn from the batch cultures immediately after addition of the spores/infected material and the later samples were withdrawn during a varying time (Table 4). From the undiluted samples and from each dilution (2.3.1 and 2.3.2 respectively), 0.1 ml was spread on agar plates, in total 10 plates per sample removed. Plates were incubated as described earlier (2.1) until colonies appeared, or for about 1.5 week. Detection limit for all trials were 10 cfu/g residue. This corresponds to one cfu in a 0.1 ml sample from undiluted residue. Therefore, even when no growth is observed there might still be viable parts, at a concentration below the detection limit.

2.3.1 Addition of spores or agar mixture

In batch trials where fungal survival was studied with spores or an agar mixture, containing fungal structures, 100 ml serum vials were used. The vials were filled, during flushing with N₂, with 10 g wet weight biogas residues and either 5 gram of the agar mixture or spore solution, containing 10⁶ spores/ml. The vials were then closed with butyl rubber stoppers and aluminum caps. The exact amount of cfu added to the serum vials, used in the batch test, were determined by serial dilution (in steps of ten) of the spore solution/agar mixture followed by plating on agar (Table 2). Several vials were filled and started simultaneously and after incubation at 37 °C duplicate vials were removed and opened at each sampling time. At each sampling occasion the whole material in the bottle was analyzed, i.e. after spreading of 0.1 ml undiluted residue on a plate, the first dilution was performed by adding 90 ml of peptone water directly to the bottle. The following dilution steps were made with 1 ml aliquots of sample to 9 ml of peptone water i.e. dilution in steps of ten.

Table 4. Experimental set-up of batch experiments used for survival tests of different fungi.

Fungi	Biogas residue^a	Fungal material^b	Number of added cfu (amount of materia added)^c	Amount cfu/g total materia^d	Incubation time (total)
<i>Aphanomyces cochlioides</i>	Norrköping	Blended OMA, (580 µm filter)	400 cfu (5 gram)	27.7	25 days
<i>Aphanomyces cochlioides</i>	Norrköping	Chopped sugar beet	Unknown (3.3 gram)	-	33 days
<i>Botrytis cinerea</i>	Norrköping	Spores	4.7 x 10 ⁶ cfu (1 ml)	4.3 x 10 ⁵	24 h
<i>Botrytis cinerea</i>	Norrköping	Chopped sugar beet	Unknown (3.3 gram)	-	33 days
<i>Botrytis cinerea</i>	Västerås	Spores	2.2 x 10 ⁶ cfu (1 ml)	2 x 10 ⁵	5 h
<i>Fusarium culmorum</i>	Norrköping	Spores	8.8 x 10 ⁶ cfu (1 ml)	8 x 10 ⁵	24 h
<i>Fusarium culmorum</i>	Norrköping	Chopped sugar beet	Unknown (3.3 gram)	-	33 days
<i>Fusarium culmorum</i>	Västerås	Spores	9.9 x 10 ⁶ cfu (1 ml)	9 x 10 ⁵	5 h
<i>Pythium ultimum</i>	Norrköping	Blended P ₅ ARP, (580 µm filter)	2750 cfu (5 gram)	183	25 days
<i>Pythium ultimum</i>	Norrköping	Chopped sugar beet	Unknown (3.3 gram)	-	33 days
<i>Rizoctonia solani</i>	Norrköping	Grinded dry barley kernels	Unknown (0.61 gram)	-	3 days
<i>Rizoctonia solani</i>	Västerås	Wet barley kernels	Unknown (0.8 gram)	-	14 days
<i>Rhizomucor variabilis</i>	Norrköping	Spores	7.6 x 10 ⁶ cfu (0.4 ml)	7.3 x 10 ⁵	22 days
<i>Mucor circinelloides</i>	Norrköping	Spores	8 x 10 ⁶ cfu (0.5 ml)	7.6 x 10 ⁵	22 days
Fungi from natural infected SSB	Västerås	Chopped sugar beet	1.2 x 10 ⁵ cfu (3.3 gram)	900	21 days

a) Biogas plant from which inoculum for the batch experiment was collected

b) Type of fungal material added to the batch cultures, used for the survival studies

c) Total number of cfu added to the batch cultures and volume or weight of the fungal material added

d) Number of cfu per g added materia (biogas residue and fungal material), added residue were either 10 g or 130 g per vial

2.3.2 Addition of grains or sugar beets

When using infected crop material for the survival studies, 309 ml serum vials were used. The vials were filled with 130 g wet weight biogas residues during flushing with N₂. In these trials the crop material was added on basis of weight and the amount corresponded to an organic load, of 4 g VS/L & day, a load typically used at biogas plants. To be able to quantify the

amount of fungi in the added crop material, a known amount of material, chopped sugar beet, dried and grinded or fresh barley kernels, was shaken with peptone water. For determination of cfu, the obtained suspension was serially diluted with peptone water, in steps of ten, and each dilution as well as undiluted samples was spread on agar plates (Table 2). The serum vials were incubated at 37 °C, on a rotating table (100 rpm), during the whole experiment. Duplicate vials were analyzed throughout the whole study by withdrawal of liquid samples after different times. The samples were removed with a syringe without opening the vials. At each sampling occasion 1.1 ml biogas residues were removed and 0.1 ml undiluted sample was spread on agar plate (Table 2). The remaining sample (1ml) was diluted with 9 ml of peptone water and the following dilution steps were then made with 1 ml aliquots of sample to 9 ml of peptone water i.e. dilution in steps of ten. When organic material is added to the batch cultures it is important to ensure that the process does not become overloaded. If too much organic material is added to the biogas system this commonly results in an accumulation of volatile fatty acids (VFA). To ensure that the biogas systems used for the trials were not overloaded the levels of VFA were investigated. Liquid samples were removed from the batch vials 5 days after the experimental start. These samples were centrifuged and the supernatant was analysed for presence of VFA by HPLC (Agilent 1100, Skandinaviska Genetec) equipped with a refractive index detector and a Rezex ROA-Organic Acid H+ (8%) column (300X7.8 mm). The eluent was 0.005 M H₂SO₄, and the flow 0.6 ml/min. If the fatty acid content exceeded 1 g/L the trials were terminated and repeated with a lower organic load. Results from experiments having too high levels of VFA are not presented.

2.4 Batch tests for determination of gas production potential of sugar beets

To determine the gas production potential from uninfected and infected sugar beets a batch test method was used. In these experiments 130 g wet weight biogas residues were transferred to 309 ml serum vials during flushing with N₂. To reduce high levels of background production of biogas, from endogenous organic material, the residue was incubated at 37 °C for approx. one week before start of the experiment. To each serum vial approximately 3.3 g of finely chopped sugar beet was added. With the approximation that the VS content of sugar beets is 18 % VS (Moglia Stenströmmer, 2007), this addition corresponded to an organic load of 4.6 g VS/L. Batch cultures without addition of sugar beet were also started in order to determine amount of background gas produced from the digestion residue solely. All vials were closed with butyl rubber stopper and aluminum caps and incubated at 37 °C on a rotary shaker (100 rpm). The amount of gas produced in the batch tests was determined by measuring the pressure (mbar) in the vials after different incubation times. By multiplying the pressure (bar) with the gas volume in the vial (cm³) and the normal gas pressure (1.01325 bar), the amount of biogas produced was then calculated. The biogas composition (methane and carbon dioxide) was determined by analyzing gas samples, taken from these vial at the same time as the pressure was measured. The methane concentration was analyzed by transferring 2 ml of gas with a syringe to a 30 ml closed vial. Vials from several such samplings were later analyzed by gas chromatography (PerkinElmer ARNEL), equipped with a Clarus 500 column (7' HayeSep N 60/80, 1/8 SF) and a FID detector. The samples were analysed at the following conditions; injection temperature, 60 °C; oven temperature, 125 °C; detector temperature, 250 °C; carrier gas, helium; carrier gas flow, 31 mL/min. Injection was performed with the aid of Headspace sampler Turbo Matrix 110. External standards with pure methane in different dilutions were used in order to produce a standard curve. The content of carbon dioxide was analyzed with a fermentation tube according to Moglia Stenströmmer (2008). After sampling, the gas pressure in the bottle was equalised to atmospheric pressure by connecting the bottle to a gas bag. The total amount of methane produced was calculated

by multiplying the total amount of biogas (determined by measuring the pressure) with the methane concentration in the sample. The amount was rectified with the temperature difference between sampling (37 °C) and analysis (20 °C) according to $(273+37)/(273+20)=1.058$. To obtain the amount of gas and methane produced from the added sugar beet, the amount of gas produced from the inoculum materials in the controls was subtracted at each sampling point.

The dry matter (DM) content for chopped fresh and stored sugar beet was determined by drying a weighed sample in an oven for 20 h at 105 °C. The samples was then allowed to cool and again weighed and the sample, left after drying, represented the dry matter content. The dried sample was then allowed to burn at 550 °C for 6 h. Material left after this burning represent the ash content while the difference in weight before and after heating to 550 °C represent the organic content, i.e. the volatile solids (VS).

To determine the effect of the fungal infection on the sugar content in sugar beet both the outer, infected, parts and the inner, less infected, parts of a stored sugar beet was analyzed on Dionex ICS-3000 equipment by Syngenta Seed AB, Landskrona, Sweden.

2.4.1 Artificially infected fresh sugar beet (FSB)

From a fresh sugar beet (FSB) stored in +4 °C a thin slice of about 30 g was cut and one of the four sugar beet pathogens were transferred either as an agar piece with growing fungi (*A. cochlioides*, *P. ultimum*) or as a spore suspension (*B. cinerea*, *F. culmorum*). The infected beet slice was then placed in a moist chamber and incubated for 2.5 week at conditions as described earlier (2.1). Thereafter, the beet slices were chopped and added to the batch culture vials according to the description in section 2.4. Two parallel bottles for each fungus were prepared. In addition, five bottles with non infected FSB as well as five bottles with no addition of sugar beet was started (Table 5). The biogas residue used as inoculum for the batch cultures in this experiment was taken from the biogas plant in Norrköping.

Table 5. Set up of batch experiment for analysis of methane production potential from fresh sugar beets (FSB).

Number of vials	Biogas residue	Substrate
5	Norrköping	-
5	Norrköping	Uninfected FSB
2	Norrköping	<i>A. cochlioides</i> infected FSB
2	Norrköping	<i>B. cinerea</i> infected FSB
2	Norrköping	<i>F. culmorum</i> infected FSB
2	Norrköping	<i>P. ultimum</i> infected FSB

2.4.2 Naturally infected stored sugar beet (SSB)

The sugar beet used in this trial had been stored for one year at +2 °C in a closed and dark box. During this time a natural flora of storage pathogens had developed. The outer parts of this sugar beet were used for determination of gas production potential from naturally infected sugar beet. The inner part of the beet was considered as representative for uninfected stored sugar beet (SSB). It was however hard to find good undamaged parts, everything was dark brown and “soggy”. The SSB was added to the batch culture vials according to the description in section 2.4. In order to compare the amount of gas produced from batch test with residue

from different biogas plants, materials from both Norrköping and Västerås where used with the same chopped sugar beet, the infected part (Table 6). Also controls with no addition of sugar beet were started. All batch tests were started in parallels of five.

Table 6. Set up of batch experiment for analysis of methane production potential from stored sugar beets (SSB).

Number of vials	Biogas residue	Substrate
5	Västerås	-
5	Västerås	Uninfected SSB
5	Västerås	Naturally infected SSB
5	Norrköping	Naturally infected SSB

2.5 Identification of unknown fungi

Two fungi previously isolated from a German biogas plant (Zetterström, 2008), and fungi appearing on plates with samples taken out from the trial with naturally infected stored sugar beet (SSB), that could not be identified by microscopically examinations, were identified using 18S rDNA-sequencing. The fungi were grown in Malt Extract broth on a rotating table at 25 °C for 48 h and the mycelium was collected from the broth by filtration. Chromosomal DNA was isolated using the Fast DNA SPIN soil kit (MP Biomedicals) and was later used as template in a PCR-reaction using PuRe-Taq PCR-beads (Amersham BioSciences) according to the manufacturer's instructions. The primers used were NL1 5'GCATATCAATAAGCGGAGGAAAAG3' and NL4 5'GGTCCGTGTTTCAAGACGG3' for fungi from naturally infected sugar beet and SR6R- AAGWAAAAGTCGTAACAAGG and LR1- GGTGGTTTCTTTTCCT for fungi appearing in biogas residue. The reaction was run as follows: 1) 96 °C for 10 sec, 2) 45 °C for 15 sec and 3) 72 °C for 1 min, repeated 30 times. The resulting PCR-product was purified using the EZNA Nucleic Acid Isolation™ Cycle-Pure Kit (Omega bio-tek). The final DNA products were sequenced at Uppsala Genome Centre, Rudbeck laboratory. The sequence obtained was blasted against all available public databases using National Center for Biotechnology Information, using nucleotide blast. [<http://blast.ncbi.nlm.nih.gov/Blast.cgi>]

3 RESULTS & DISCUSSION

3.1 Survival of sugar beet pathogens

The result showed that within 48 h all viable structures tested was killed. Even within 22 h all fungi tested (*Fusarium culmorum*, *Botrytis cinerea*, *Pythium ultimum*, *Aphanomyces cochlioides*, *Rhizomucor variabilis* and *Mucor circinelloides*) were below or just above the detection limit. The fastest reduction was seen for *F. culmorum* and *B. cinerea* that were reduced from 10⁵ cfu/g residue to less than 10 cfu/g within 2.5 h. Furthermore, to ensure that the fungi were not just inhibited but actually killed all fungi, except *Rhizoctonia solani* (due to contamination), were tested also for a longer period of time (25-33 days, table 4). This incubation time represent a common HRT for a mesophilic continuous biogas process. If just inhibited the fungi could possibly adapt to the environment and on a later stage start growing again. However, no survival fungi was seen after longer incubations times suggesting that they were either irreversibly inhibited or indeed killed.

Most fungi produce some sort of survival structures. The survival structure for *Aphanomyces cochlioides* and *Pythium ultimum* are oospores, *Rhizoctonia solani* produce sclerotia while *Fusarium culmorum*s survival structures are chlamydospores. *Botrytis cinerea* can produce both chlamydospores and sclerotia. As these structures are tolerant towards extreme environmental factors their survival would have been interesting to test in the anaerobic batch reactors. Unfortunately, this was not possible due to the difficulty in obtaining these structures on artificial media. The survival studies in anaerobic batch reactors were instead carried out with mycelia, oogonia and conidia. The lack of survival structures in the batch experiments makes it difficult to completely predict the fate of these fungi in a biogas process.

3.1.1 *Aphanomyces cochlioides*

The OMA mixture added to the residue contained 80 cfu/g which corresponded to 27 cfu/g residue (5 g OMA mixture to 10 g residue). In the first sample, removed after incubation for 1 h, the amount had been reduced to 5 cfu/g. In the second sample removed after 3.5 h, no viable fungi were detected, suggesting that they were quickly killed in the environment prevailing in a biogas process. The OMA mixture added in this experiment had been filtered in several steps why it is unlikely that any zoospores were present. Furthermore, as no oospores could be detected during microscopy the fungal structures added to the batch cultures were likely only mycelia and oogonia. In the beginning of this study, as in the experiments with *P. ultimum* (3.1.2) where agar was added, mycelia seemed to be growing on the surface of the residue in the vial. Still, no fungal growth was observed when the material was plated on CMA. It is thus unclear what was actually seen in the bottles and if this surface structures really was fungal growth. In the second experiment, when *A. cochlioides* had been pre-grown on sugar beet slices, the number of cfu added were not determined and no growth was visible on the sugar beet slice. Thus it is not surprising that no cfu were detected after plating the sample removed immediately after addition of the beet to the batch cultures. Therefore it is possible that no viable fungal structures were added to the residue together with the sugar beet slices. So, from these experiments it is not possible to predict the survival of *Aphanomyces cochlioides* in a biogas reactor. Oogonia and mycelia seem to die rapidly but naturally infected sugar beets probably also contains oospores that could not be produced in this study and thus, have not been tested. The most reliable results would probably be obtained if naturally infected sugar beet could be used as inoculum for fungal structures.

3.1.2 *Pythium ultimum*

The P₅ARP mixture added to the residue contained 550 cfu/g, which corresponded to 183 cfu/g residue (5 g P₅ARP mixture to 10 g residue) in the batch cultures. However, already in the samples removed after incubation of 1 h, no viable fungi could be detected. As with the OMA mixture with *A. cochlioides* it is unlikely that any zoospores were present and that the only structures added to the batch cultures were mycelia and oogonia. No oospores were seen during microscopy. In the second experiment, when *P. ultimum* had been grown on sugar beet slices, the number of cfu added were not determined. However, from the sample removed immediately after addition of the slice to batch cultures, 3.4×10^4 cfu/g residue was detected. In the second sample, removed after 26 h, no growth was observed. It is not known which fungal structures that gave rise to cfu at time zero. However, it is not likely that any oospores had been produced during the 2.5 weeks of incubation with the sugar beet. Conclusively, these studies performed with *P. ultimum* cannot completely predict the survival in a biogas reactor. Oogonia and mycelia seem to die rapidly but with the results from these experiments it is not possible to predict the fate of oospores. To clearly predict the fate of *P. ultimum* naturally infected sugar beet, with developed oospores should be tested.

3.1.3 *Rhizoctonia solani*

Rhizoctonia solani was tested several times. Unfortunately contamination occurred at each test, probably by *Penicillium* species. So, even when *R. solani* was shown to be present on the kernels no growth of it was observed in samples taken out from the batch cultures. These results can be explained by a high sensitivity of *R. solani* to the environment in the biogas system, thus quickly disappearing, or that it was out competed by the quick growing *Penicillium* on the agar plates. In the experiment only mycelia were added as no production of sclerotia seem to have taken place. Neither was it possible to establish an infection with *R. solani* on sugar beet. This lack of results makes it impossible to speculate about the survival of this fungus in a biogas plant.

3.1.4 *Botrytis cinerea*

The survival of *Botrytis cinerea* was investigated in two experiments after addition of conidia to the batch cultures. Approximately 4.7×10^6 cfu/g was added to the residue, which corresponded to 4.3×10^5 cfu/g (1 ml (1 g) spore suspension to 10 g residue) and no viable fungi were detected after 2.5 h, which was the first measuring point (Figure 1). In the survival experiment with *B. cinerea* grown on sugar beet slices (FSB), the number of cfu added was not determined. Analysis of sample removed immediately, after addition of the sugar beet to the batch cultures, demonstrated 620 cfu/g residue. However, in the second sample, after almost 26 h of incubation, no growth was observed after plating. Apparently, this fungus was very quickly killed, independent source of inoculum (spore suspension or infected sugar beet). The quick reduction obtained in this study is supported by Marquenie et al (2002) showing that with conidia in a spore suspension heated in a glass capillary the decimal reduction time was 30 min at 40 °C. Conclusively, if material infected with *B. cinerea* was taken into a continuously feed biogas process there would most likely remain no viable fungi in remaining digestion residue, unless new material and residue would be taken out and added with shorter intervals than 2.5 h.

3.1.5 *Fusarium culmorum*

Also *F. culmorum* seemed to be reduced quickly in the biogas plant environment. Spores were added as 8.8×10^6 cfu/g, corresponding to a concentration of 8×10^5 cfu/g residue and after incubation of 2.5 h no growth could be observed (Figure 1). However, after 4.5 h, the fungi seem to reappear as 20 cfu/g residue was obtained after sampling liquid from from one of duplicate bottles. The sample in the other bottles did not show presence of any surviving fungal structures. It is possible that the fungus had survived in some sheltered place, as some heterogeneity in the biogas environment may exist. The results may also have been caused by a contamination during removal and plating of the sample. In the sample removed after 6.5 h or later in the study, no fungal growth was detected. The reduction speed is thus very fast for conidia from *F. culmorum* as there were 8×10^5 cfu/g from the start and none alive after 6.5 h of incubation. This was confirmed in a second trial where approximately the same amounts of spores were added and in the first sample, after 5 h, no growth was seen. Conclusively these experiments suggest a very fast reduction of *F. culmorum* in a biogas process. Similarly, when *F. culmorum* was added as infected sugar beet the first sample, removed immediately after addition of material to the batch bottles contained 2.5×10^3 cfu/g residue. In the second sample, after almost 26 h of incubation, no growth appeared. It can thus be concluded that the risk for survival is very small for conidia or mycelia from *F. culmorum* in a biogas plant. If

entering a biogas process these structures would most likely die off between two feedings, in a continuously feed reactor, or otherwise likely disappear in the post digestion tank.

3.1.6 *Rhizomucor variabilis* and *Mucor circinelloides*

These fungi were isolated in residue from a biogas plant in Hohenberg-Krusemark, Germany (Zetterström, 2008). They were identified through sequencing of 18S rRNA gene. For fungus 1, 486 of 491 bases were identical to *Rhizomucor variabilis*. For fungus 2, 629 of 634 bases were identical to *Mucor circinelloides*. The identification needs to be confirmed with other techniques since there were several base pair mismatches for both strains. Also, no rhizoids were observed for *R. variabilis*, which makes the identification questionable.

Both fungi were tested in separate survival tests. The concentration of *R. variabilis* spores were 7.3×10^5 cfu/g residue in the batch cultures (19×10^6 cfu/ml added, 0.4 ml/vial with 10 g residue). At the first sampling, performed immediately after addition of the spores, the detected level were 3.4×10^5 cfu/g residue. The second sample, removed after 5 h of incubation, contained 4.8×10^4 cfu/g residue (Figure 1). After 22 h of incubation, 10 cfu/g residue was detected in the sample from one of the parallel bottles. In the sample taken from the other parallel bottle, no growth was detected.

The *M. circinelloides* spores were added to a final concentration of 7.6×10^5 cfu/g residue (16×10^6 cfu/ml added, 0.5 ml/vial, 10 g residue). The first sample, removed immediately after addition of the spore suspension to the bottles, contained 7.3×10^5 cfu/g residue (Figure 1). In the second sample, removed after 5 h of incubation, the number had been reduced to 20 cfu/g residue in the sample from one of the two flasks and none in the parallel bottle. After 22 h of incubation, no fungi were detected. However, in the fourth sample, after 28 h, 3 colonies grew in the undiluted sample from one of the two flasks, none from the other. As this might have been a contamination, the trial should be repeated to verify the survival time. No fungal growth was observed in samples removed later.

These results are based on one single experiment and they must be repeated to clearly verify their fate in a biogas plants and its residues. However, the results indicate that *R. variabilis*, and possible *M. circinelloides*, survived for a longer period of time than any other fungus tested in this study. This might be the explanation to why they were found in residue. Both *R. variabilis* and *M. circinelloides* is frequently found around the world in soil, plants and decaying fruits and vegetables but they can also cause diseases in animals and humans. It is therefore possible that these fungi appeared in the biogas residue as they were present in the in-going material and not just due to contamination of the residue in a later stage.

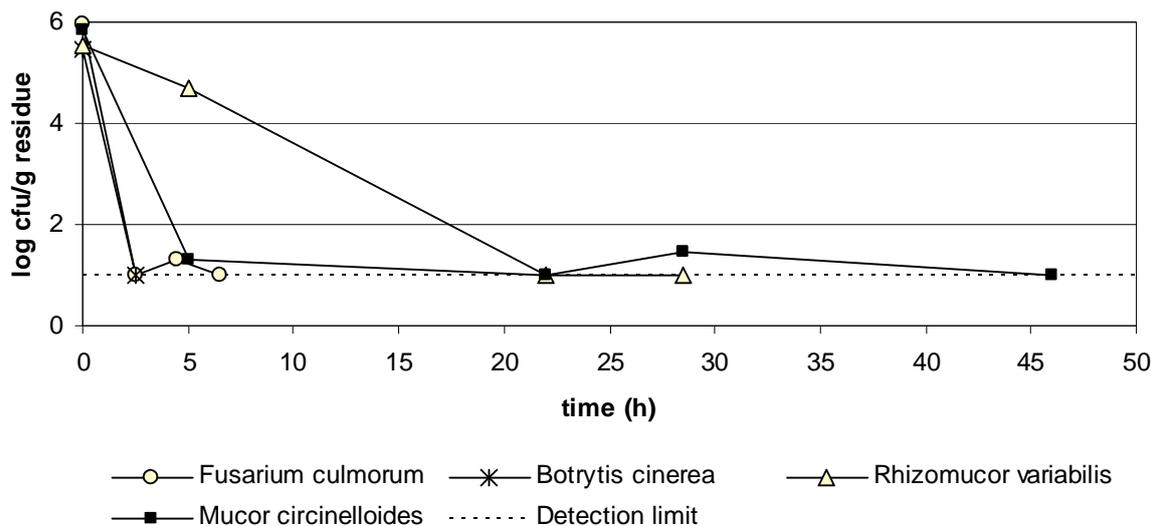


Figure 1. Survival curves for fungal spores in batch cultures started with biogas residue from Norrköping biogas plant. The detection limit is 10 cfu/g residue. All curves represent single values from one of the duplicate bottles. This was done because if the mean value was to be used there would be values below detection limit, and this is not possible. Each fungus is shown only until no growth is detected anymore, the sampling occurred for a longer time (see table 4).

3.1.7 Pathogens on naturally infected sugar beet

The added naturally infected SSB contained 3.3×10^4 cfu/g, this corresponds to 937 cfu/g residue (3.3 g added, 130 g residue). The samples taken out were spread on both PDA and MEA/ox to detected different fungi. Both in the sample spread from added material and the first sample taken out, at 0 h containing 2.6×10^3 cfu/g, and the second sample after 4.5 h with 8 cfu/g, the only fungi appearing on both MEA/ox and PDA was *Penicillium* spp. The *Penicillium* spp. were identified through sequencing of the 28S rRNA gene. Three different colonies were identified further, one from MEA/ox and one with white and one with yellow mycelia from PDA. The result from sequencing showed that all three were 99-100 % identical both to *Penicillium commune* and *Penicillium expansum*, 550 of 550, 586 of 586 and 584 of 585 bases. It can therefore, through the sequencing, not be concluded exactly which specie the tested colonies actually was. *P. commune* is mostly a contaminant on cheeses, meat products and other food products (CBS, 2008). *P. expansum* on the other hand grows on fruits, household waste, meat and in soil (CBS, 2008). Independent of which *Penicillium* spp. they were quickly reduced in the biogas process. After 4.5 h of incubation they were reduced from 2.6×10^3 cfu/g to 8 cfu/g. After 24 hours of incubation no growth for this fungus was observed.

In the sample taken after 24 h, another fungus appeared as one colony in one serie, corresponding to 3 cfu/g residue (4 plating series). Also in next sample, at 45 h, 3 cfu/g residue was detected. DNA was extracted from these colonies and they were both identified by sequencing of the 28S rRNA. The obtained sequences were both 100% identical to the ascomycete *Pseudallescheria boydii* over a region of 576 base pairs. *P. boydii* is frequently found as saprobe in agricultural soil, manure and polluted water but it may also infect weakened humans (CBS, 2008). As this fungus is present in soil on plant roots at harvesting it is likely that it can be added to the biogas digester together with the sugar beet. However, this

would probably not cause any problems as it is frequent in soils and does not seem to be pathogenic towards any crop.

After 13 days of incubation another fungus appeared, corresponding to an amount of 3 cfu/g undiluted sample. Sequence analysis showed that this fungus was identical (577 of 578 bases) to the ascomycete *Byssochlamys nivea*. The natural habitat of this fungus is food, such as grain and especially fruit, but it can also be found in soil (CBS, 2008). As with *P. boydii* it is likely that this fungus could enter a biogas plant due to soil following the sugar beet at harvest. The fungus is not a known plant pathogen in field so it would likely not cause any problems. It has probably entered the biogas digester together with soil on sugar beet.

3.2 Gas and methane production

3.2.1 Fresh sugar beet

When fresh sugar beets were used as substrate in the batch tests, gas production immediately started and continued for a very long time (Figure 2). Even after 90 days of incubation, when the experiment was terminated, the biogas production had not levelled off. This is much longer than a normal HRT of a continuous biogas process. Also, it took about one month until the level of methane in the produced biogas reached 50-60 % of the total amount of gas produced. This means that in a continuous biogas process with a retention time of 20-25 days, the methane yield would be only about one fourth of the total potential.

The biogas and methane accumulation curves from infected and uninfected fresh sugar beet (FSB) followed each other. However, during the whole incubation period the uninfected sugar beet produced more biogas (and methane) than the infected beets (Figure 2). Here, it should be noted that the standard deviation was greater for the infected sugar beet than for the non infected beets. This was probably due to a high heterogeneity of the added material caused by a varying degree of infection.

The biogas and methane production curve from infected sugar beet is shown as a mean value of the gas or methane produced from beet infected with the four different fungi tested, i.e. from in total eight bottles (Figure 2). It is good to view the infected sugar beets as replicates of each other as damaged sugar beets likely are infected not by one but by several different fungi.

A large difference was seen when the gas production from sugar beets infected with the different fungi was compared. The beet infected with *A. cochlioides* gave the highest levels of biogas and the beet infected with *F. culmorum* gave the lowest amount (Table 7). The difference in biogas production was probably not so much attributed to different fungi as it was to the level of fungal infection. For *A. cochlioides* there was no visible infection on the sugar beet slice, when it was added to the batch cultures, and no growth was detected in the samples taken out immediately after adding the infected sugar beet to the batch culture. The degree of infection was higher for *F. culmorum*, also giving a high cfu value at start. The amount of cfu/ml in the zero samples is rather well correlated to the amount of gas and methane later produced during 90 days of incubation (Table 7).

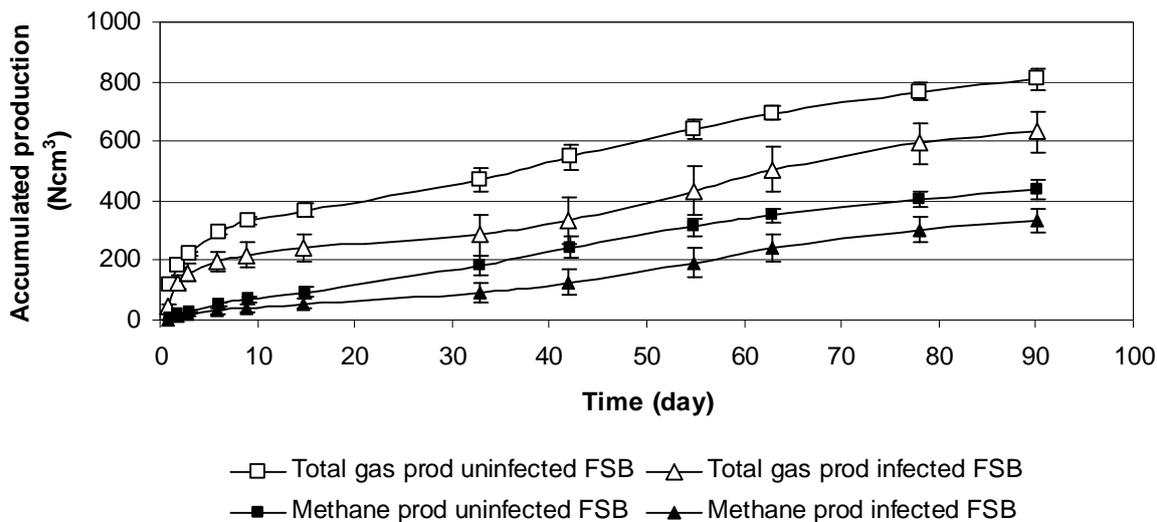


Figure 2. Amount of biogas and methane produced in batch cultures with pathogen infected and uninfected fresh sugar beet (FSB) as substrate. The accumulated production is standardized to zero degrees and one atmosphere. The biogas and methane production from the infected sugar beet, in each time point, is an average of in total eight samples, i.e. duplicate samples from four different fungi. Background biogas and methane production from endogenous material in the inoculum material (residue from Norrköping biogas plant) is subtracted.

Table 7. Fungal cfu/ml in the first (“zero”) sample, taken out just after start of batch cultures with infected sugar beet slices, and the total gas- and methane production measured after 90 days of incubation. Total gas and methane production is standardized to zero degrees and one atmosphere. The value for uninfected sugar beet (SB) represents a mean value from 5 bottles. Background biogas and methane produced from endogenous material in the inoculum (residue from Norrköping biogas plant) is subtracted from the biogas and methane values from batch cultures with the infected sugar beet.

Fungi	cfu/ml	Total gas production (Ncm ³)	Methane production (Ncm ³)
Uninfected SB	-	809	439
<i>A. cochlioides</i> 1	0	711	378
<i>A. cochlioides</i> 2	0	720	381
<i>B. cinerea</i> 1	600	688	371
<i>B. cinerea</i> 2	640	608	324
<i>F. culmorum</i> 1	2 500	547	290
<i>F. culmorum</i> 2	2 400	578	313
<i>P. ultimum</i> 1	32 000	633	345
<i>P. ultimum</i> 2	36 000	565	284

3.2.2 Stored sugar beet

The batch cultures, with addition of one year stored sugar beet (SSB), produced most of the biogas during the first 25 days (Figure 3). After 25 days the biogas (and methane) production levelled off and the methane concentration in the biogas reached 50-60 % of the total biogas. This results indicate that in a continuous biogas process with a “normal” HRT of 20-25 days, most of these sugar beets would have been degraded and converted to methane.

Also in this experiment the biogas and methane accumulation curves from infected and uninfected sugar beets followed each other. There was no significant difference in average gas

production for the uninfected sugar beet as compared to the infected SSB. However, during the whole experiment, the level of methane produced from the uninfected sugar beet was significantly higher than from the infected beet (Figure 3).

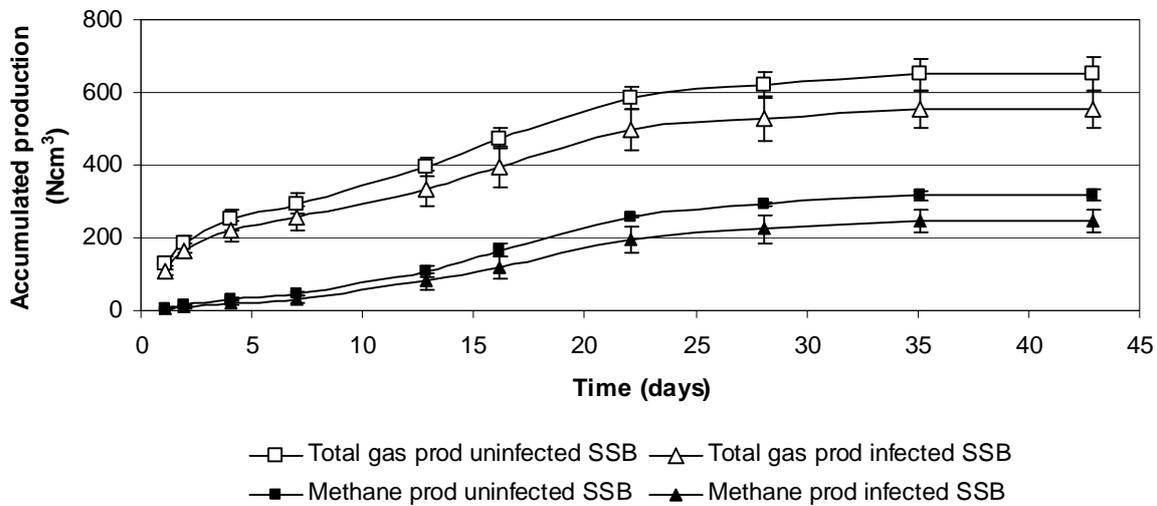


Figure 3. Amount of biogas and methane produced in batch cultures with pathogen infected and uninfected stored sugar beet (SSB) as substrate. The accumulated production is standardized to zero degrees and one atmosphere. The biogas and methane produced is an average from five replicates per time point. Background biogas and methane production from endogenous material in the inoculum material (residue from Västerås biogas plant) is subtracted.

The same infected SSB was tested in batch cultures started with inoculum from either Västerås or Norrköping biogas plants. The amount of gas and methane produced from the sugar beet were the same independent of residue, at least for the 42 days of incubation used in this experiment (Figure 4). This result suggests that it is likely that the difference in gas production from fresh sugar beet, in batch cultures started with residues from Norrköping, and stored sugar beet, in batch cultures started with residues from Västerås, is due to differences in the sugar beet substrate and not due to differences in the residues. However, the standard deviation for the gas production when Norrköping residue was used was much greater than when the batch cultures were started with residue from Västerås biogas plant.

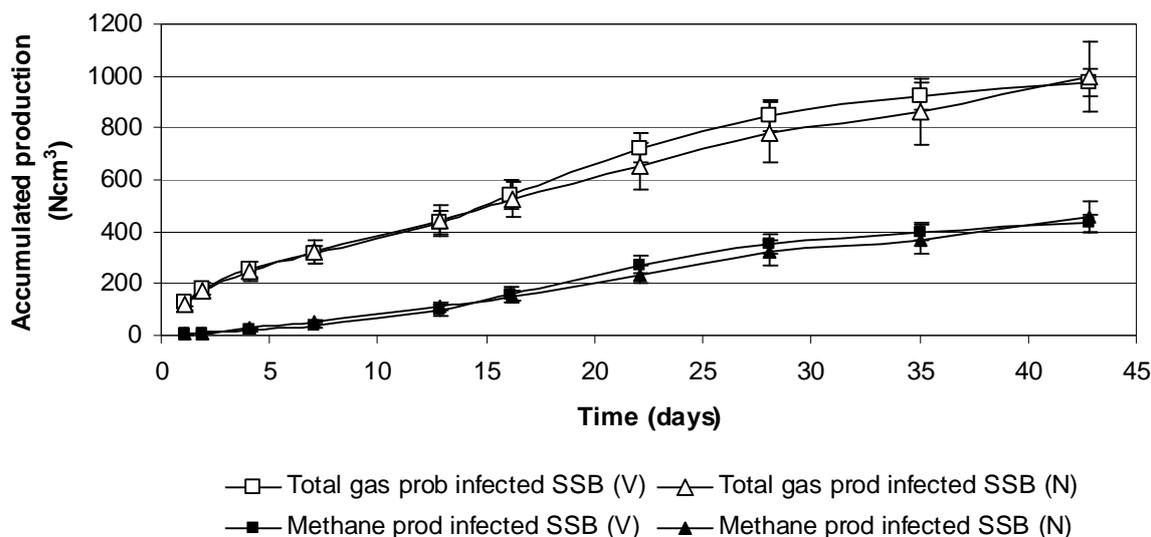


Figure 4. Amount of biogas and methane produced in batch cultures started with residue from either Norrköping (N) or Västerås (V) biogas plant and with pathogen infected stored sugar beet (SSB) as substrate. The accumulated production is standardized to zero degrees and one atmosphere. The biogas and methane values are an average from five replicates per time point. Background biogas and methane production from endogenous material in the residue from the different biogas plant is not subtracted.

3.3 Comparison between gas trial

Big differences in the total amounts of methane produced in the two experiments, with fresh or stored sugar beet, could be seen both at end and after 25 days of incubation (Table 10-11). As different source of inoculum did not give any difference in gas production (Figure 4) it can be concluded that the difference in gas and methane production from SSB and FSB most likely is caused by differences in the sugar beets. The amount of biogas and methane was lower from infected sugar beet than from uninfected, independent of beet storage time. This result can be explained by the fact that the fungi use carbon as energy source for its growth, thereby giving less carbon available for methane production. A consumption of sugar due to infection was confirmed with a sugar analysis (Table 8). This analysis demonstrated that the total amount of sugar was lower in infected SSB than in uninfected SSB. In the infected SSB some saccharose had been converted into other sugars, but the total amount of sugar was still lower in the infected SSB compared to uninfected SSB. Since this analyse was performed on the inner, uninfected, and outer, infected, parts of the same sugar beet it is likely that the observed loss was due to fungal infections and not just due to variations in the same beet. The fungi use carbon in sugars for respiration and growth. This lower sugar, and thus carbon content, is probably the reason for lower methane production from infected SB as compared to infected.

Table 8. Sugar content in uninfected and infected stored sugar beet (SSB).

	Glucose g/100g SB	Fructose g/100g SB	Saccarose g/100g SB	Total sugar g/100 g SB	Total sugar g/g VS
Uninfected SSB	0.42	0.57	21.33	22.31	2.60
Infected SSB	1.76	2.09	13.80	17.65	2.05

The stored and fresh sugar beet was added assuming that both had a volatile solids (VS) content of 18 %. However, when the sugar beets were analysed it was clear that neither of them actually had this VS content (Table 9). During storage of sugar beet the dry matter (DM) and VS content had decreased. The decrease in DM suggests that the water content has increased during storage, which seems unlikely. The decrease in DM could possibly instead have been caused by a transformation of some carbon into volatile acids such as acetate. During the dry matter analysis acids can, due to their volatility, evaporate from the sample and thus giving a underestimated DM value. The reduction of the VS content was likely due to both natural respiration from the sugar beet, lowering the sugar content, and by growth of storage pathogens and possibly also due to the loss of organic acids. Therefore, the organic load per litre was not, as assumed, 4 g VS/L but instead, according to VS analysis, 2.5 and 7.3 for SSB and FSB, respectively. The VS analysis suggests that the FSB was added at a too high organic load. The carbon in sugar beet is in a readily available form as sucrose and this would also mediate a quick degradation by the first microbial groups. This together might have inhibited the process through an increase in VFA. As the level of VFA was not measured in this trial it is only a speculation that possibly can explain the comparably extended gas production curves for FSB. For SSB, most of the gas and methane was produced during the first 25 days of incubation but for FSB only about 30 % of the total production occurred during this time (Table 10-11).

Table 9. Average values from determination of dry matter (DM) and volatile solids (VS) in four samples each of fresh and stored sugar beet (SB) and actual organic load added per litre in the batch cultures used for determination of gas production potential.

	DM (%)	VS (%)	Organic load g/L
Fresh SB (average)	30.15	29.50	7.49
Fresh SB (stand.dev.)	1.52	1.59	-
Stored SB (average)	9.41	8.59	2.23
Stored SB (stand.dev.)	0.17	0.20	-

There are a great differences in the amount of methane produced per g VS between FSB and SSB (Table 10-11). According to these results it seems better to store sugar beets for one year before anaerobic digestion. The methane yield in this experiment, for SSB, was also much higher than the 450 m³ CH₄/ton VS (equal to cm³/g VS) found in literature for anaerobic digestion of sugar beet with tops (Biogassysds homepage, 2008). However, this apparent high methane yield can possibly be due to loss of carbon during DM and VS determination due to volatilization of organic acids (see above). If so the real VS is higher than the value (0.28-0.29) used for the calculations (Table 10-11). When instead calculating the amount of methane produced per g wet weight (WW) the values are more reasonable and most methane is produced from the uninfected FSB, as would be expected.

Table 10. Amount of biogas and methane produced (Ncm³), totally and after 25 days of incubation of batch cultures with pathogen infected and uninfected stored (SSB) and fresh sugar beet (FSB). The batch cultures were started with residue from either a biogas plant in Norrköping (N) or in Västerås (V). Background production from endogenous organic materials in the inoculum is subtracted. The accumulated production is standardized to zero degrees and one atmosphere.

	Uninfected FSB (N)	Infected FSB (N)	Uninfected SSB (V)	Infected SSB (V)
Total g VS¹	0.97	0.97	0.29	0,28
Total g WW²	3,3	3,3	3.4	3,3
Total biogas (Ncm³)	809 (36) ³	631 (67,7) ⁴	651 (46,2) ³	552 (52,1) ⁴
Total biogas (cm³/g VS¹)	834	651	2260	1916
Total biogas (Ncm³/g WW²)	245	191	194	166
Biogas 25 days (Ncm³)	420	280	600	510
Biogas 25 days (Ncm³/g VS¹)	433	289	2085	1821
Biogas 25 days (Ncm³/g WW²)	127	85	179	154
Total CH₄ (Ncm³)	439 (30,4) ³	336 (39) ⁴	318 (14,7) ³	245 (31,4) ³
Total CH₄ (Ncm³/g VS¹)	453	346	1105	858
Total CH₄ (Ncm³/g WW²)	133	102	95	74
CH₄ 25 days (Ncm³)	150	90	300	210
CH₄ 25 days (Ncm³/g VS¹)	155	93	1034	750
CH₄ 25 days (Ncm³/g WW²)	45	27	90	63

¹ VS= Volatile Solids

² WW= Wet Weight

³ Standard deviation between 5 parallels

⁴ Standard deviation between 8 parallels

The table below (Table 11) shows relative amount of gas and methane produced. The uninfected FSB is set to 100 % as this was expected to produce more gas than the other treatments. The FSB digestion had a very elongated gas production phase, it had not ceased when the experiment was terminated after 90 days. That is the reason why the SSB have a higher gas and methane production after 25 days than the FSB (Table 11). The slow start and elongated gas production could possibly be explained, as mentioned above, by an excessive addition of sugar beet. The addition was made according to VS value in literature that later proved to be incorrect for the used sugar beet. The high gas and methane production for both SSB per g VS is possible due to a loss of carbon during DM determination and is thus not likely values. It is more accurate to compare the total amount produced or when calculating per g wet weight.

Table 11. Relative amount of gas and methane produced, totally and after 25 days, after incubation in batch cultures with pathogen infected and uninfected stored (SSB) and fresh sugar beet (FSB). The batch cultures were started with residue from either a biogas plant in Norrköping (N) or in Västerås (V). Background production from endogenous organic materials in the inoculums is subtracted. The production from uninfected FSB is set to 100 %.

	Uninfected FSB (N)	Infected FSB (N)	Uninfected SSB (V)	Infected SSB (V)
Total biogas (Ncm ³)	100	78	80	68
Total biogas (Ncm ³ /g VS ¹)	100	78	271	230
Total biogas (Ncm ³ /g WW ²)	100	78	79	68
Biogas 25 days (Ncm ³)	100	67	143	121
Biogas 25 days (Ncm ³ /g VS ¹)	100	67	482	421
Biogas 25 days (Ncm ³ /g WW ²)	100	67	141	121
Total CH₄ (Ncm ³)	100	74	72	56
Total CH₄ (Ncm ³ /g VS ¹)	100	76	244	189
Total CH₄ (Ncm ³ /g WW ²)	100	77	71	56
CH₄ 25 days (Ncm ³)	100	60	200	140
CH₄ 25 days (Ncm ³ /g VS ¹)	100	60	667	484
CH₄ 25 days (Ncm ³ /g WW ²)	100	60	200	140

¹ VS= Volatile Solids

² WW= Wet Weight

4 CONCLUSIONS AND FUTURE RESEARCH

From the results in this study the potential problem with fungi entering a biogas plants can not be fully evaluated. It turned out to be more difficult than expected to obtain spores from the plant pathogenic fungi. Survival structures of fungi are formed when the plant tissue start to collapse, which is far from the situation on nutrient rich agar medium. Survival structures can often withstand extreme environments such as heat, low oxygen and toxicity. It would therefore been of interest to test their survival. The mycelia, conidia and oogonia tested for different fungi in this study were mostly killed very rapidly. As none of these structures are produced to survive harsh environment the result is not surprising. However, as most material used in a biogas plants must be stored it might be of more interest to see the fate of storage pathogens rather than that of plant pathogens. Particularly as the plant pathogens, being pathogenic fungi often needing living tissues, might not survive storage. Storage pathogens on the other hand are specialized on mature and decaying tissue and might thus be present in greater number after storage.

Both in this study and in the study by Zetterström (2008) all storage pathogens tested were reduced below detection limit within two days of incubation. The storage pathogens tested in this study, *F. culmorum*, *B. cinerea* and *Penicillium* spp., was all reduced very fast from high initial numbers to not detectable within 5 h of incubation. In a continuous biogas plant this might still lead to problem as residues are taken several times every day. The residue taken out is however further degraded in a post-digestion tank and later also kept in an aerobic storage compartment before used as fertilising agent. Depending on the prevailing environment in these tanks, the fungi can either continue to decrease in number or start to grow. Schnürer & Schnürer (2006) studied the behaviour of six different fungi during the post digestion aerobic storage and reported that the numbers of all tested fungi were further reduced. They speculated that the reason might be the limited access to carbon sources. At a biogas plant commonly four steps can be identified, pasteurization, anaerobic digestion, post-digestion and aerobic storage. All these different steps represent different environments and conditions which the fungi must survive if to be present in the bio manure and later spread in the field. It seems unlikely that a fungus can be adapted and survive all these steps and manage the change in environmental conditions. Therefore the risk with plant pathogens in the biogas plant seems low. This study has however not been able to completely confirm or deny that. The behaviour of fungi during the entire chain, not only during the anaerobic digestion, must be further studied in order to completely evaluate risk for fungal survival. Furthermore, during such studies it is important to include different fungal species as they are likely to react differently if spread on the field. Some fungi are very host specific and might only survive in fields in the presence of the right crop while others have a wider host range or can survive on organic material in the soil. Some fungal structures are also capable of surviving for very long period of times in the soil, while others die more quickly. These factors, among others, will also influence the level of risk and it is therefore difficult to draw general conclusions about fungal behaviour and problems if they are spread on fields.

In future studies, especially of *Aphanomyces cochlioides* and *Rhizoctonia solani*, it would be optimal to use naturally infected sugar beet. These are more likely, as compared to sugar beets infected by man, to contain relevant fungal survival structures and also relevant amounts of each pathogen. *Pythium ultimum* does not naturally infect the sugar beet root and will possibly be more difficult to investigate. However, in this study it was shown that this fungus could be grown on sugar beet slices. Possibly it can also be inoculated onto a sugar beet. The fungi detected in biogas residues from Germany, *Rhizomucor variabilis* and *Mucor circinelloides* seemed to survive a bit longer than other fungi tested. In future studies it might be of interest to test the biogas residue once more to check if it was an unfortunate contamination when taking out the residue or if they actually are present in the biogas process. The species isolated from naturally infected sugar beet *Penicillium* sp. died within 24 h but the two fungi discovered in later samples, *Pseudallescheria boydii* and *Byssoschlamys nivea*, needs to be further investigated in survival trials in order to be completely evaluated.

If fungi on infected crop material are killed in the biogas process, anaerobic digestion offers a good way to use this material. However, it is then important to consider that, as shown in this study, fungal infected material give rise to less gas, and methane, than uninfected material. In both gas trials, the infected material produced about 20 % less total methane/g VS than non infected sugar beets. The lower level of gas is caused by fungal growth, using carbon in the sugars for respiration. This needs to be taken into account when calculating the gas potential for different substrates and the economy of a biogas plants digesting fungal infected material. To completely reveal the gas loss caused by fungal infection it is important to test also other substrates and other infecting fungi. Even though less gas is produced, biogas production still

seems like a good way to dispose organic material unsuitable for other production. At least through that the material would generate something positive for the environment through creation of energy and mediating a recirculation of nutrients.

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APPENDIX 1 – MEDIA*Malt ekstrakt agar with ox bile*

H ₂ O	1 l
Malt ekstrakt agar	50 g
Ox bile (Merck)	10 g
Autoclave	

Potato Dextrose Agar (PDA) with cholesterol

H ₂ O	1 l	
Potato dextrose agar (Oxoid)	39 g	
Autoclave		
Cholesterol	1,33 ml	(15 mg/ml EtOH)

Ox bile media

H ₂ O	100 ml
Barley kernels	100 g
Autoclave for 1h, wait 24 h add another 100 ml H ₂ O, autoclave 1h, repeat the procedure once more after another 24 h.	

H ₂ O	1 l
Ox bile (Merck)	1 g

Autoclave

Pour 100 ml ox bile/jar with barley kernels, transfer 10 pieces of growing *R. solani* mycelia and incubate for 24 days.