Innovative New Fungal Products Produced with Solid Substrate Fermentation

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Abstract: The mushroom industry has a long history of using solid substrate fermentation for the production of spawn and growing substrates. Our laboratory is engaged in a number of projects designed to expand our use of this core technology beyond the mushroom industry. Production of several new products such as Red Yeast Rice, spore forming biopesticides including the mycoherbicide, Smolder,™ and the production of specialty chemicals for use in the agriculture, food and pharmaceutical sectors will be discussed. In addition we are combining the use of solid substrate fermentation with recombinant DNA technology in order to develop the cultivated button mushroom as a platform for the production of additional unique products.

Key words: Red Yeast Rice, *Alternaria destruens*, zearalanone, *Agaricus*, transformation

1 Introduction

Solid substrate fermentation can be defined as "the growth of microorganisms on moist solid materials in the absence of free flowing water."[1, 2] The mushroom industry has a long history of utilizing this technology, first in the preparation of the growing substrate (composting), and later in the production of uniform inoculum (spawn making).

A large variety of substrates have been used in solid substrate fermentation, including rice, wheat and other forage materials, grains, beans and corn. Several "non-traditional" substrates have also been utilized in the industrial process as well. These include industrial and food processing wastes, such as sawdust and wheat bran, as well as particulate materials including vermiculite, clays, paper pellets, and even sponge-like materials. When compared to submerged fermentation processes, the advantages of using solid substrate fermentation include:

- Higher yields when compared to submerged culture
- Growth conditions are more similar to the native environment of the organism under exploitation
- The opportunity is provided to insure uniform dispersion of the inoculum throughout the medium
- The relatively low moisture environment may favor the production of specific compounds or structures (i.e. spores) which may not be produced or are produced poorly in a submerged environment
- Because the substrate is relatively concentrated, solid substrate fermentation normally requires less space than submerged fermentation to contain equal amounts of substrate.

As one might expect there are also disadvantages to the solid substrate approach as compared to a submerged liquid system:

- Only those organisms which can grow at relatively low moisture levels can be used
- It is difficult to determine the amount of biomass produced
- Due to its solid nature, it is difficult to accurately measure process parameters such as pH, oxygen, etc.
- Typically, processing times are longer than submerged systems.

In the late 1970's, Sylvan developed patented technology[3] that allowed for the production of large amounts of sterile solid substrates, which could be inoculated and mixed aseptically (Fig. 1). Aliquots of the inoculated

substrates could then be delivered into specially designed incubation bags that allowed for the axenic growth under largely uniform, defined conditions (Fig. 2). This system reduced the dependence on capital-intensive equipment and increased the flexibility of the solid substrate fermentation platform at a relatively low cost.

Figure 1. Sylvan's solid substrate fermentation system
Preparation, sterilization and inoculation of solid substrates are carried out in a single vessel.

Figure 2. Bags filled aseptically with inoculated substrate. The filled bags then serve as individual fermentation vessels

For the last 10 years, our laboratory has been engaged in utilizing this technology beyond the realm of mushroom spawn production in order to determine whether new uses could be commercially exploited. Production of biopesticides; production of secondary metabolites; and production of pharmaceuticals through the use of recombinant DNA technology are three potential areas of exploitation that we have investigated. This presentation will detail some of the research carried out at the laboratory in an effort to expand the uses of commercial solid substrate fermentation technology.

2 Fermentation of *Alternaria*

In 1984 a new fungus, later identified as *Alternaria destruens* (Fig. 3), was discovered on diseased dodder (*Cuscuta spp.*) in Wisconsin, USA.[4] Dodder, a seed producing parasitic weed, is an obligate parasite that penetrates the host plant and absorbs nutrients from the phloem. It is found as a pest in cranberry, carrot and potato production, among others. It can be particularly destructive in cranberry bogs, where it causes reduced plant vigor, significant yield losses and reduced berry quality (Fig. 4). In 1997, we were approached to determine whether or not our solid substrate fermentation system would be suitable for the commercial production

of *Alternaria destruens* spores.

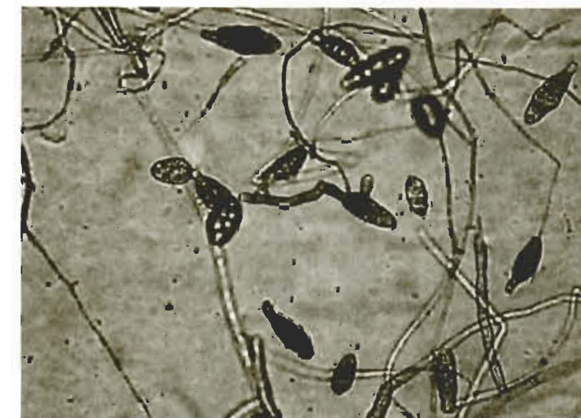

Figure 3. Spores of the fungus *Alternaria destruens*

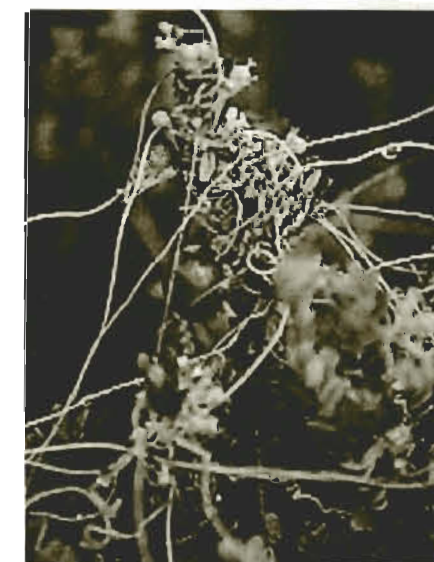

Figure 4. Dodder (*Cuscuta* spp.) growing on cranberries in Wisconsin, USA

Our laboratory began by going back to the field with our collaborators and isolating additional fresh isolates of A. *destruens* on dodder. After fulfilling Koch's postulates in the greenhouse, the most promising strains were subjected to a series of laboratory studies in order to determine their suitability for inclusion in our fermentation system. These studies included: determining the type and level of nutrients required for successful growth and sporulation; the effect of light and dark including the optimum photocycle, and finally, the appropriate levels of oxygen required to optimize the system and minimize the deterioration of the maturing spores. The strain selected for commercialization was then subjected to DNA fingerprinting analysis to ensure that it could be identified in the field. The obtained fingerprint also served as the basis for monitoring the strain's stability over time.

Our objective in designing an acceptable production system was to maximize the yield of *Alternaria* spores on a minimum volume of substrate, such that the spores that are relatively small (25-40 x 10-13μm) could be efficiently separated from the substrate on which they were grown. Because of our history of growing fungal materials on grain based substrates such as rye and millet, these and similar materials such as rice and soybean were tested. However, while these substrates were able to sustain good mycelial growth, they were unable to produce economically acceptable quantities of the spores. Following further experimentation and discussions with experts in the field, it was determined that the selected culture preferred high levels of cellulose as a food

source. Growth of A. *destruens* on a cellulose-enriched substrate resulted in significantly higher levels of spore production with a particle size amenable to separation. Separation of the spores from the substrate was then achieved by the use of grinding followed by a cycle of cyclonic separation (Fig. 5).

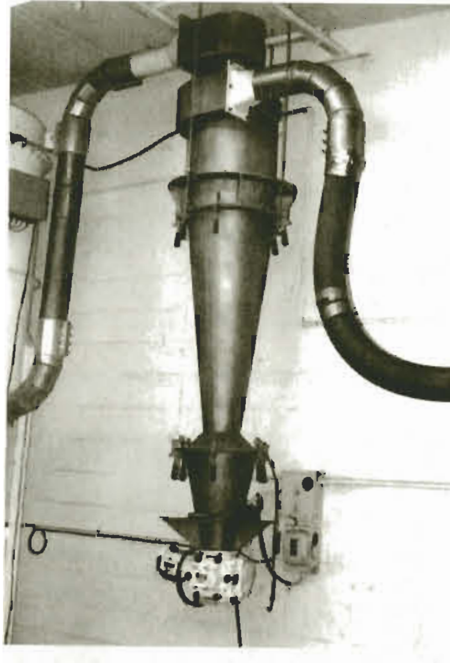

Figure 5. Equipment used to separate dried and ground A. *destruens* from their growing substrate

With the use of this technology, yields in excess of 1x 10^{10} spores/gram of finished product were achieved. The material produced in this manner provides economically acceptable levels of dodder control (Fig. 6). An added benefit is that, following separation, enough viable *Alternaria* remain on the cellulose-enriched substrate that the "waste" material is suitable for use as an early season pre-emergent dodder control product. Both the post emergent spore based product and the pre emergent cellulose based product are currently awaiting final approval by the US Environmental Protection Agency for use on a variety of crops including cranberries, carrots and potatoes.

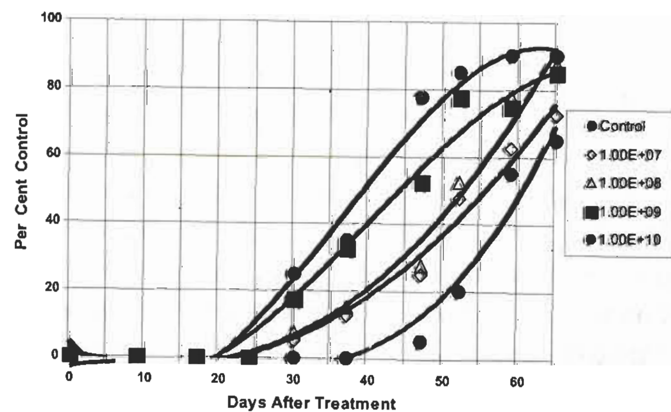

Figure 6. Effect of spore concentration on Dodder Control Carrot Study, Wisconsin

3 Fermentation of Zeranol

An example of how solid-state fermentation technology can be used to develop pharmaceutical grade specialty chemicals, can be illustrated in the development of zeranol.

Zeranol is an non-steroidal estrogen analog used in sheep and cattle production to stimulate growth and optimize grain consumption, thus leading to increased animal protein content.[5] Commercial use of zeranol began in the 1960's and is administered via implantation to more than 25 million animals annually (Ivy Laboratories, personal communication).

The active ingredient is produced via reduction from zearalenone, a compound that is produced in nature by *Fusarium graminearum,* a common pathogen of corn (Fig. 7). A series of zearalenone producing isolates were obtained and subjected to evaluation in small-scale laboratory fermentations. The strains were grown using a variety of liquid media, which was adsorbed onto vermiculite, an inert solid substrate. Optimum temperature, time, and respiration characteristics were included in the experimental matrix. Process efficiency was measured as the percent conversion of glucose to zearalenone. Based on the percent conversion, two strains, S028 and S073 were selected for commercial development. These strains were capable of producing greater than 4 grams of zearalenone per 100 grams of glucose, in less than 30 days in laboratory studies (Fig. 8).

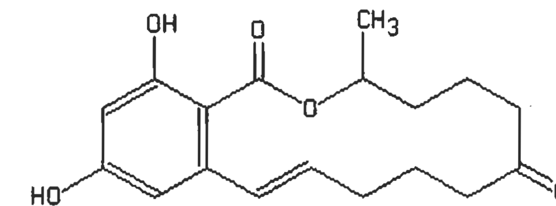

Figure 7. Structure of zearalenone

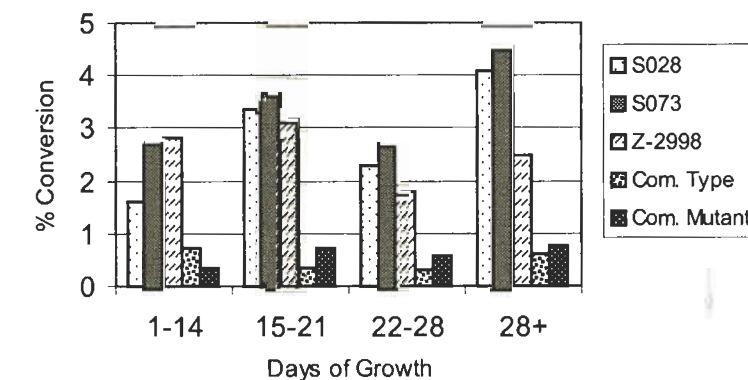

Figure 8. Conversion (in percent) of glucose to zearalanone of five *Fusarium graminearum strains*, grown for at least 28 days

Due to its high absorbing capacity and inert nature, vermiculite, supplemented with a complete liquid, high glucose, nutrient media continued to be the preferred solid substrate. Grown in this manner, yields were optimized and zearalenone could be efficiently extracted from the colonized substrate using a variety of methods, without compromising the integrity of the target molecule.

Development of the process technology from the laboratory scale to commercial production technology involved defining bulk raw materials, bulk substrate preparation protocols and the optimization of commercial inoculum preparation. It was found that laboratory grade reagents including glucose did not perform in a manner identical to their bulk industrial grade siblings. Therefore materials from a variety of commercial sources were tested both in the laboratory and at the pilot scale level before a final "recipe" could be defined and approved. Similarly, strain maintenance and inoculum production procedures needed to be identified before a stable and reliable production system could be put in place.

4 Red Yeast Rice

Another product, which successfully exploits solid substrate fermentation technology, is Red Yeast Rice, a

product that has been produced in Asia for centuries.[6] Traditional culture is carried out using rice that is cooked, drained and then inoculated with a fungus of the genus *Monascus*. The inoculated substrate is allowed to ferment on the ground, typically in cool, damp rooms that have been lined with fresh wet clay soils. The fermented product is dried and used for a variety of food and medicinal uses including winemaking, food coloring and for the improvement of blood circulation[7, 8]

In the past several years, Red Yeast Rice has become a popular dietary supplement in non-Asian countries. However, production of a safe, reliable product that conforms to the Good Manufacturing Practices (GMP) standards established in many countries was difficult using the traditional production system. Several suppliers of dietary supplements approached Sylvan and asked us to (a) develop a production system and (b) supply a product that would stand up to the scrutiny of the regulatory system and thus meet consumer demands.

In order to adapt the traditional production to Sylvan's unique fermentation technology, we began by screening a large number of strains of several *Monascus* species, both wild and domesticated. Strains were cultured on a variety of substrates including rice, soy and wheat. However, as the product is known as red yeast rice, we evolved to a rice-based substrate supplemented with small amounts of other nutrients. We compared our results to those found in products produced using traditional Chinese fermentation systems by HPLC analysis (Fig. 9).

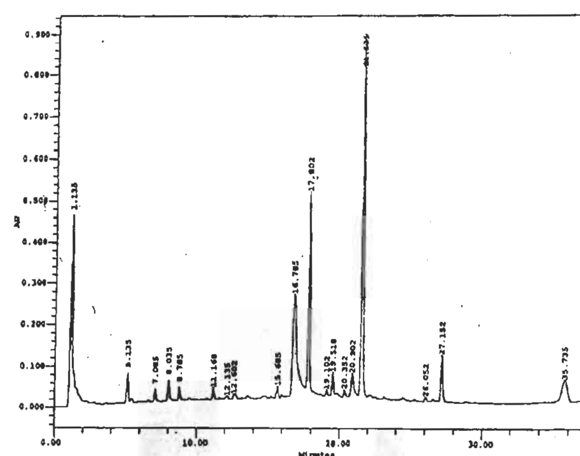

Figure 9. HPLC Traces of Red Yeast (RYR)
Conditions: gradient elution, Detection: UV detect, Ca 237nm, Flow Rate: 1 ml/min. (Li, pers. comm.)

Red Yeast Rice contains a number of bioactive compounds including mono-, di- and polyunsaturated fatty acids, phytosterols, isoflavones, pigments and terpenoid compounds including HMG-CoA Reductase inhibitors.[9] One such terpenoid, Monacolin K, was discovered in 1979 by Endo.[11] The compound blocks the synthesis of cholesterol in the liver by inhibition of a rate limiting reaction within the biosynthesis pathway.[12] In addition to Monacolin K, at least eight related monacolins have been identified as the products of Red Yeast Rice fermentation.[13]

A screening system was developed based on the native levels of monocolins found in the isolates that we examined. A strain with a bioactive compound spectrum that closely matched traditional fermentation when grown in Sylvan's proprietary system was selected. The strain of *Monascus purpureus* is fermented on organic rice in a batch production system for several weeks. It is then dried, ground and sterilized before it is sold either in bulk or as formulated capsules. As with other Sylvan products, molecular markers are used to insure that the strain remains stable over time.

5 Exploitation of Transformed *Agaricus*

Potentially the most far ranging use of solid substrate fermentation may be in the development of its use as a

non-animal, high value, drug production platform. Commercial mushroom farming routinely produces biomass of 30-40 kg/m^2 over a period of 8-10 weeks. Using the *Agaricus bisporus* transformation system described by Chen et al,[13] our laboratory undertook a demonstration project designed to determine whether the button mushroom of commerce could be used to produce heterologous proteins of interest.

We selected a patented *Agaricus bisporus* strain, J1901, for our work because it has the unique characteristic of being a sporeless variety, thus limiting the potential for unintended release or movement of the recombinant germplasm. Using the *Agrobacterium* mediated system described above, genes coding for two therapeutic proteins, alpha-galactosidase A (used in the treatment of Fabry's syndrome) and somatropin (human growth hormone) were introduced into tissue blocks of strain J1901 (Fig. 10).

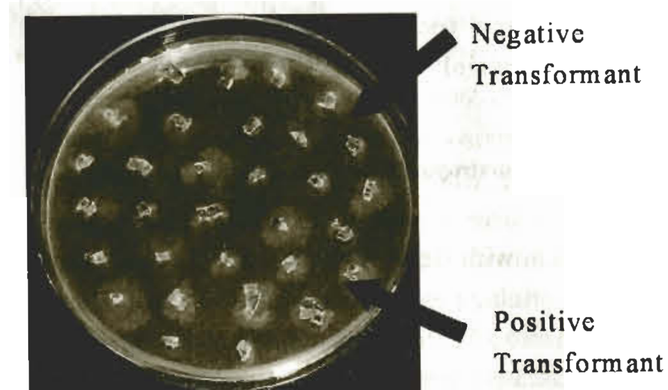

Figure 10. *Agaricus bisporus* tissue blocks show positive and negative transformants

Successful transformation events were monitored by resistance to hygromycin and then assayed via Southern analysis (Fig. 11). Alpha-galactosidase a activity was measured spectrophotometrically, using 4-nitrophenyl-α-D-galactopyranoside as a substrate. In the case of somatropin transformations, presence of the gene sequence was performed by PCR analysis in addition to verification by Southern analysis (Fig. 12). Detection of the gene product was carried out using commercially available antibodies obtained from Roche Diagnostics.

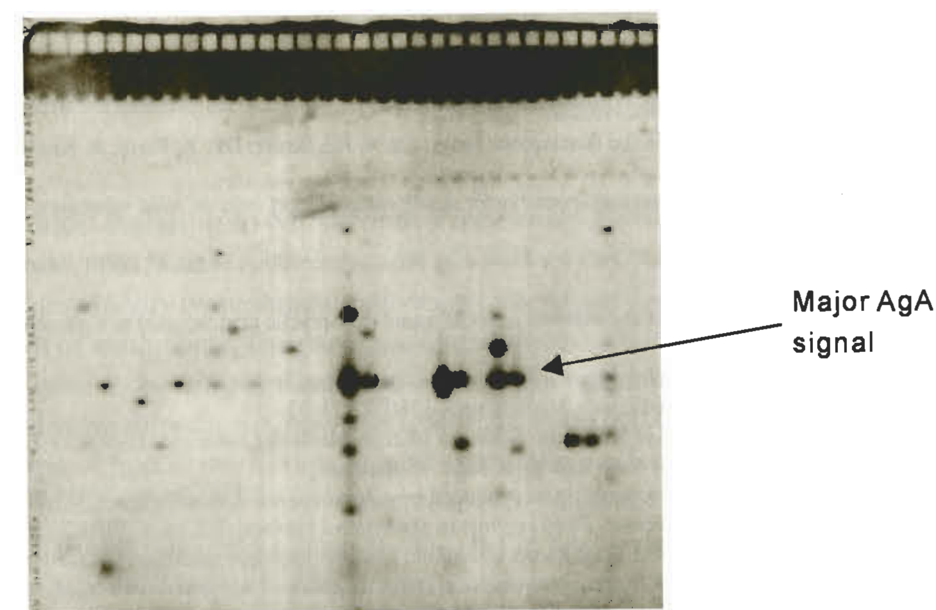

Figure 11. Southern hybridization of AgA transformants showing positive signals for alpha-galactosidase a

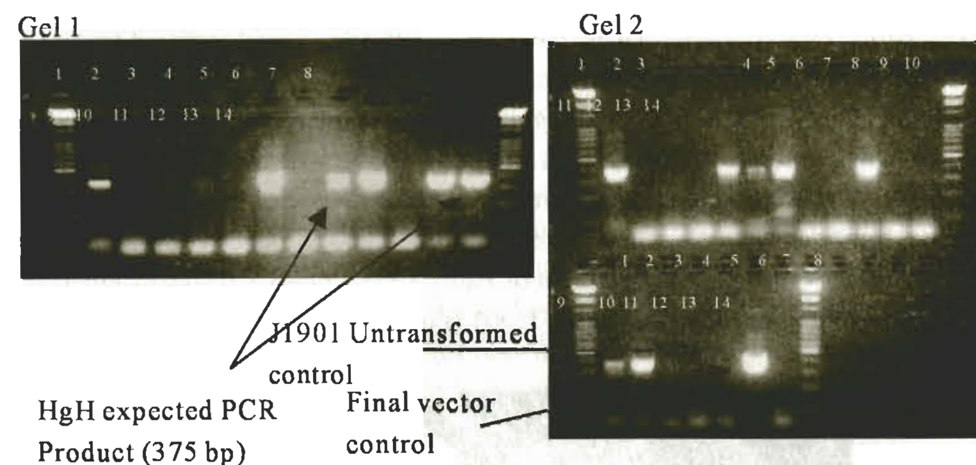

Figure 12. PCR analysis of putative somatropic transformants shows the expected 375 amplification product

These results have led to collaboration with Bayer Crop Science designed to demonstrate the utility of this technology for the production of aprotinin, a therapeutic protein used in open heart surgery. Transformation vectors were designed, transformed into J1901 and assayed for the expression of recombinant aprotinin in mushroom fruiting bodies in approximately 6 months. Initial expression levels of 0.1% total soluble protein were obtained, and current work is directed at improving the levels of recombinant protein expression in this system.

6 Conclusions

These examples are to illustrate the utility of solid substrate fermentation technology for the production of a wide variety of commercial products. As the interest in fungal-based products continue to grow, more opportunities to exploit this technology should present themselves, allowing organizations with strong backgrounds in the areas of fungal genetics, nutrition and physiology to thrive.

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